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13. ABSTRACT (Maximum 200 Words) Thyroid hormone (T3) is essential for skeletal growth and development. Excess T3, especially in adults, can cause bone loss and increased fracture susceptibility. Our previous data showed that T3 increases local production of a critical bone growth factor, insulin-like growth factor-I (IGF-I), and potentiates interleukin-1 (IL-1)-stimulated production of the osteoclastogenic cytokine interleukin-6 (IL-6). The current proposal was to determine whether these local factors are important in the effects of T3 to stimulate osteoblast proliferation and to generate bone-phenotypic proteins, i.e. alkaline phosphatase, osteocalcin, collagen, and in the resorptive effects of T3. The data obtained support these conclusions: antibody, antagonist peptide or antisense oligonucleotide to the IGF-I receptor prevented the anabolic effects of T3, and antibody to the IL-6 receptor inhibited the bone-resorbing effect of T3. A second goal was to investigate the mechanisms by which T3 stimulates production of IGF-I and IL-6. T3 had small stimulatory effects and enhanced the effect of IL-1 to increase IL-6 mRNA and IL-6 promoter construct expression. T3 had small stimulatory effects on IGF-I mRNA expression. Thus, transcriptional effects contribute to the actions of T3 to increase both local factors. T3 interacted with PTH, increasing PTH receptor expression and augmenting PTH signaling in osteoblastic cells.				
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INTRODUCTION

The **subject and purpose** of our study was to determine the mechanisms by which excess thyroid hormone (T3) causes bone loss and increased susceptibility to fractures. This is an important problem since hyperthyroidism is a relatively common disease and also because thyroid hormones are a frequently prescribed medication, both appropriately and inappropriately. Also, there is the potential for excessive thyroid hormone intake through non-proprietary remedies. Although this has been more tightly regulated during recent years, such products are still available. T3-mediated bone loss represents a particularly interesting problem because T3 action is also critical for the formation of bone. This is particularly true during development, but may also be maintained later in life and obscured by effects on pathways that become amplified as the individual matures and ages. This duality of effects is probably not unique to T3. A number of agents initially identified as growth factors, are now recognized as having actions to promote bone resorption. Parathyroid hormone is now recognized to have important anabolic activity in addition to its well-recognized catabolic effects. Thus, understanding thyroid hormone effects on bone may help us to understand the actions of a number of other factors on the skeleton as well as providing elucidation of the effects of this clinically and pharmacologically important factor itself.

An important insight into how these dual effects could arise derived from the observations that T3 can increase the production of local factors in bone, and that these factors included both important bone growth factors and critical mediators of osteoclastogenesis. These preliminary data indicated that T3 increased the production of the bone growth factor insulin-like growth factor-I (IGF-I) and promoted the production of the inflammatory osteoclastogenic cytokine interleukin-6 (IL-6). It was our hypothesis that these factors were critical for the anabolic and catabolic effects of T3 on bone, respectively. Testing this hypothesis this hypothesis defined the part of the **scope** of the research. Specific Aims IB and IIB were designed to determine this. The other aspect of the research was to elucidate the mechanisms by which thyroid hormone regulates or modulates the production of the local factors (Specific Aims IA and IIA). These studies could provide a framework for understanding how thyroid hormones would affect bone in a given individual and how these effects might be modulated.

BODY

The Specific Aims and the Annual Goals of the Statement of Work were as follows:

IA: Determine the mechanism of the effect of T3 to increase IGF-I in osteoblastic cells

IB: Determine the biological significance of the increased IGF-I for the anabolic effects of T3

IIA: Determine the mechanism and modulation of T3 potentiation of IL-6 production

IIB: Determine the biological significance of the T3 potentiation of IL-6 production

Year 1:

(IA) Optimize conditions for studies of T3 stimulation of IGF-I secretion in osteoblastic cells; begin measurement of IGF-I mRNA by RT-PCR in osteoblastic cells; begin IGF-I promoter analysis

(IB) Produce antisense oligonucleotides to IGF-IR; begin to test effects of excess IGFBP3 on anabolic effects of T3

(IIA) Select optimal human bone cell model for further studies of T3-enhanced IL-6 production; begin measurement of IL-6 mRNA by RT-PCR (IIA)

(IIB) Optimize conditions for studies of interactions of T3 and IL-1 on resorptive parameters

Year 2:

(IA) Begin studies on T3 effects on the synthesis of IGF-I protein; continue analyses of T3 effects on IGF-I mRNA; begin studies of IGF-I mRNA half-life/rate; continue IGF-I promoter analysis

(IB) Determine effects of IGF-IR antisense oligonucleotides on effects of T3; complete studies on effects of excess IGFBP-3 on anabolic effects of T3; begin studies of antisense IGF-IR cDNA on T3 actions

(IIA) Continue studies of IL-6 mRNA; begin studies on IL-6 half-life/rate; begin IL-6 promoter analysis

(IIB) Begin studies of effects of IL-6 neutralizing antibodies on resorptive actions of T3

Year 3:

(IA) Complete studies on T3 effects on synthesis of IGF-I protein; complete studies on T3 effects on IGF-I mRNA; complete studies on T3 effects on IGF-I mRNA half-life/rate; IGF-I promoter analysis

(IB) Complete studies of antisense IGF-IR cDNA on T3 effects

(IIA) Complete studies of IL-6 mRNA half-life/rate: complete IL-6 promoter analysis; compare effects of T3 with those of other hormones that affect IL-6; investigate interactions of T3 with other resorptive hormones and cytokines on IL-6 production

(IIB) Complete studies of IL-6 and IL-6R neutralizing antibodies on T3 effects; investigate interactions of T3 with other hormones and cytokines on resorptive parameters

We have carried out the proposed studies. Also, there have been some findings from other studies that impact on the overall goal of the understanding the actions of thyroid hormones on bone, and these are included at appropriate points in the report.

Specific Aim IA:

Specific Aim IA was designed to follow up on our published studies (1) showing that T3 increased IGF-I in bone organ cultures and osteoblastic cells. The Specific Aim was to determine the mechanism of the effect. As reported in the 1st year Progress Report we demonstrated specific binding of ¹²⁵I-T3 to the human osteoblastic cell lines HOS and MG63 and provided evidence for interaction with both α and β isoforms. In other studies, we have documented binding of ¹²⁵I-T3 in ROS 17/2.8 and UMR-106 rat osteoblastic cells and MC3T3-E1 osteoblastic cells.

We have studied the regulation of IGF-I production by thyroid hormone. We found small changes in UMR-106 cells and Saos-2 in IGF-I mRNA in response to T3. Data from the Saos-2 cells using RT-PCR was provided in the year 1 Progress Report. In RT-PCR analyses in ROS 17/2.8 and human cells a small effect of the same magnitude was also seen. During the current

year we have carried out further studies in UMR-106 cells using Northern blotting (Figure 1). Osteoblast cultures were treated with thyroid hormone (T3) 0.01 nM - 1 μ M for 8, 24 or 48 hr. When total RNA was isolated and analyzed for IGF-I mRNA by Northern blotting using an IGF-I specific riboprobe, we observed a maximal increase of approximately 2-fold, which could account for the similar magnitude of stimulation we observed on the secretion of IGF-I by UMR106 cells (Lakatos, et al. J. Bone Miner. Res. 8: 1475-1481, 1993. There was a dose-dependence of the effects, similar to that seen on IGF-I production by the cell cultures. In contrast to the effects in the cell cultures, the response was not attenuated at the higher concentrations of T3. This finding suggests that other effects are introduced by the higher concentrations of T3 which affect the production or stability of the IGF-I protein. The biphasic effect on IGF-I protein was not prevented by protease inhibitors, nor was it related to interference by IGFBPs in the assay. Thus, the attenuation resulting with the higher T3 concentrations may be at a translational or post-translational level. The attenuation could be physiologically relevant, as higher concentrations of T3 could result in the predominance of catabolic effects on bone, due to the decline in expression of IGF-I protein.

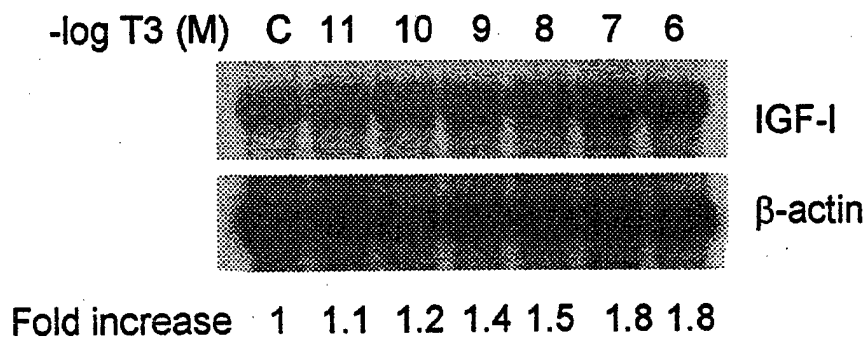


Figure 1. Dose-response effect of T3 (48-hr treatment) on IGF-I mRNA in UMR-106 cells. Total RNA was analyzed by Northern blotting and sequential hybridization with 32 P-labeled antisense transcripts specific for IGF-I and β -actin. IGF-I mRNA levels were quantitated by densitometry and normalized to β -actin mRNA.

In other studies conducted during the current year with a no-cost extension of the grant, we have analyzed IGF-I promoter activity using a series of human IGF-I promoter-luciferase fusions in UMR-106 cells (Figure 2). Gene fusions included human IGF-I promoter regions from -4770, -1208, -1033, -834, -506, -450, -184, and -126, and rat IGF-I promoter region -450 base pairs with respect to the ATG translation start site (these having been kindly provided by T. Owen, Pfizer Central Research, Groton, CT). These results suggest that T3 does not directly regulated IGF-I production at the level of transcription activation. Differences in the profile and level of transcription factor activity between human and rat osteoblastic cells may account for the lack of an observable transcriptional response to T3 in this heterologous system. We have also observed that treatment of osteoblastic cells with cycloheximide prior to the addition of T3 enhances the production of IGF-I by these cells (data not shown). One interpretation of this result is that T3 alters the activity of a putative repressor of IGF-I transcription, thus leading to an

increase in IGF-I production. It is also possible that T3 may stimulate steady-state levels of IGF-I mRNA by increasing mRNA stability. Therefore, both transcriptional and post-transcriptional mechanisms may be involved in the regulation of IGF-I production.

As described in the 1st year progress report, T3 downregulated IGF-I receptors in MC3T3-E1, ROS 17/2.8 and UMR-106 cells, indicating that IGF-I mediated anabolic effects of T3 (as documented in the studies carried out under Specific Aim IB, below) are mediated through an increase in IGF-I production, rather than through effects to increase IGF-I binding, and that the approaches we initially proposed to carry out on Specific Aim IA were appropriate and important.

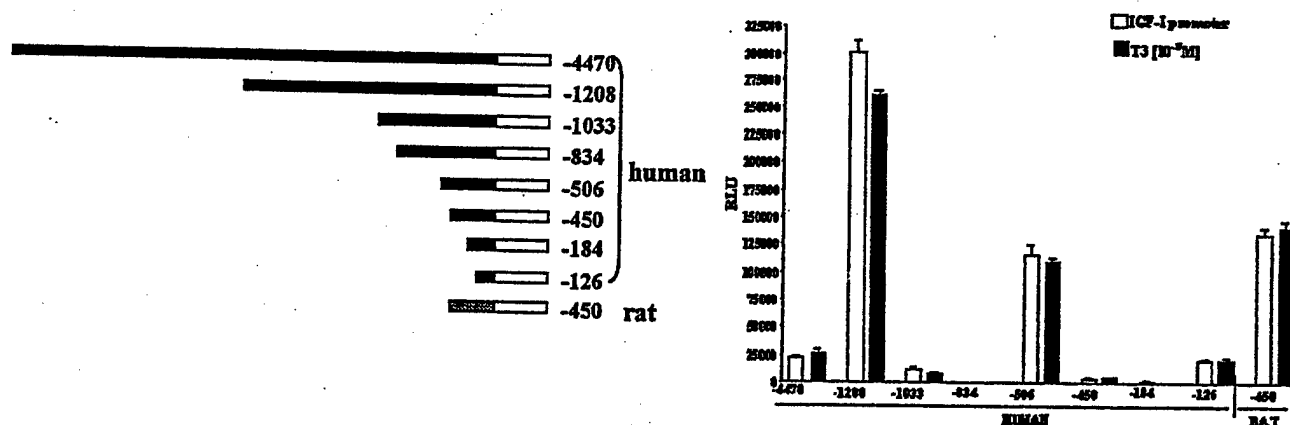


Figure 2. Effect of T3 (10^{-8} M) on human IGF-I promoter activity in UMR-106 cells. Cells were transiently transfected with promoter-luciferase reporter transgenes, treated with T3 for 24 hours, and assayed for luciferase activity.

Specific Aim IB:

Specific Aim IB was designed to determine the role of IGF-I in the anabolic effects of T3. We have carried out all of the studies outlined in the Statement of Work related to Specific Aim IB. Data from these studies was presented in the first and second year Progress Reports. Several abstracts have been published describing aspects of the work (2-4), and a manuscript (5) has been published (Huang, et al. J. Bone Miner. Res. 15: 188-197, 2000). Copies of these abstracts were included in last year's progress report; a copy of the manuscript is included in the Appendix to this final report. We have used three independent approaches to inhibit the action of IGF-I and have shown that these approaches inhibit the proliferative and phenotypic anabolic effects (osteocalcin, alkaline phosphatase, proline incorporation) of T3 on osteoblasts. The approaches used to prevent IGF-I effects were an antibody (α IR3) to the IGF-I receptor, an antagonist peptide (JB1), and an antisense oligonucleotide directed at the IGF-I. A control antibody had no effect. It was proposed to use an excess of IGF binding protein, however, the preliminary experiments utilizing this approach indicated that the amounts that would be required made the

costs prohibitive. An interesting observation was that although IGF-I stimulated cell proliferation, the growth factor alone was ineffective in stimulating the effects on the phenotypic anabolic parameters. This appears inconsistent with findings from previous studies on IGF-I (6-9). One important difference between our studies and the previous work was that our medium contained serum that had been stripped with Dowex AF-1-X-10 to remove thyroid hormones. It appears likely from our results that this procedure removes other factors that are required for the expression of the IGF-I effect and that these other factors are stimulated by thyroid hormone treatment.

Our findings from the studies under Specific Aim IB indicate that IGF-I is critical for the anabolic effects of T3, and suggest that an age-related decline in this growth factor could be an important switch to suppress the anabolic effects of the hormone and allow the expression of the catabolic actions.

Specific Aim IIA:

Specific Aim IIA was designed to determine the mechanism by which T3 potentiated the effects of IL-1 to increase IL-6 in bone organ cultures (10), a finding from our laboratory that constituted a preliminary result leading to this proposal. In studies reported in the Progress Report from the first year of the grant, we showed that although potentiation could be demonstrated in some cell lines, this was not a universal effect in all cell lines, although effects in the MG-63 cells reflected a potentiation, which was also seen when IL-6 mRNA was assessed by RT-PCR. In the first year Progress Report we showed that T3 did not increase IL-1 receptor expression in the MG-63 cells, but rather decreased the expression of the receptor. This indicated that the potentiating effects of T3 on IL-1 stimulated IL-6 secretion were not mediated at the level of the expression of the IL-1 receptor.

During the third year, we extended our studies of the mechanism by analyzing the actions and interactions of T3 and IL-1 on the IL-6 promoter. Constructs representing the full-length IL-6 promoter (-779) and shorter sequences (-224, -158, -109) coupled with a luciferase reporter were transfected into UMR-106 cells using Lipofectamine Plus®. Other cell lines (MC3T3, ROS 17/2.8, TSA) and other transfection reagents (soybean liposomes, calcium phosphate, DOTAP, Superfect®, Lipofectamine®) were tested, but not found to be satisfactory. A recent experiment indicates that Lipofectamine 2000® may be effective. The report from the 3rd year illustrated the response elements in the IL-6 promoter and showed that IL-1 had stimulatory effects in each of the constructs tested. The greatest effects, and an augmented effect in combination with T3 was found with the shortest promoter construct tested, which contains the NF- κ B response element. In other studies with the -224 promoter, shown in the third year report, interactions between T3 and another stimulator of resorption, PTH were examined. T3 and PTH effects were additive or greater. The report also showed the finding that estrogen decreased the effects of T3, PTH and T3 +PTH, however in the presence of T3, estrogen had less of an inhibitory effect on the response to PTH. Also shown in the previous report were findings obtained in a study in which RT-PCR was used to examine effects of IL-1 and T3 on IL-6 mRNA expression in MC3T3-E1 cells. There were stimulatory effects with both T3 and IL-1, and there was the suggestion of potentiation.

Specific Aim IIB:

Specific Aim IIB was designed to test the hypothesis that the effect of T3 to potentiate IL-6 production is important for the bone-resorbing effects of T3. In studies reported in the first year Progress Report we showed that an antibody to the IL-6 receptor attenuated the resorptive effects of T3. These results were reported and are included in an abstract (2). We have carried out studies to determine, by Western blotting, if T3 increases the expression of Osteoclast Differentiating Factor (ODF/RANKL/OPGL/TRANCE) in UMR-106 cells. These studies failed to show any effects of T3 on this pathway. Since IL-1 increases ODF expression in human osteoblastic cells (11), future experiments will determine if T3 potentiates the effects of IL-1 on this resorption-related parameter. As reported previously, as another parameter of resorptive activity, we attempted to measure the proteolytic activity in the media from the organ cultures by a new fluorometric assay (EnzChek®, Molecular Probes), but the method proved to be insufficiently sensitive for the culture media, although satisfactory standard curves were obtained. We have recently established a bone marrow/osteoblast co-culture model of osteoclastogenesis in our laboratory, which includes measurement of tartrate-resistant acid phosphatase as one parameter. T3 alone failed to elicit significant stimulatory effects in this model, whereas IL-1 elicited marked stimulation. In future work, T3 interactions will be investigated in this model.

To extend the studies to T3 interactions to other resorptive agents we have carried out studies on parathyroid hormone. As reported last year, we observed that 48 hr pretreatment with PTH or T3 mutually up-regulated each other's receptors in ROS 17/2.8 osteoblastic cells, and that pretreatment with T3 enhanced the calcium signals elicited by PTH. As described above (Specific Aim IIA), T3 enhanced PTH effects on IL-6 production. This suggests that the T3/PTH interaction, like the T3/IL-1 interaction, may be another mechanism by which thyroid hormone promotes bone loss. These findings have been extended further. The stimulatory effects of T3 were observed after 48 hr of culture. Scatchard analysis (Figure 3) showed that T3 increased [¹²⁵I]-PTH binding sites by approximately 38%, whereas the binding affinity was not significantly altered. RT-PCR analysis revealed that the level of PTH receptor transcripts was increased 44% (Figure 4).

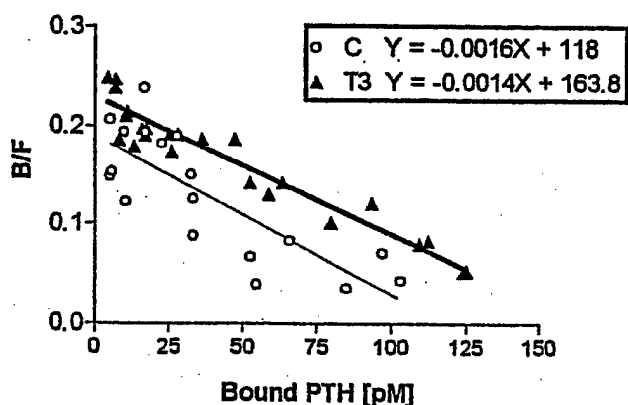


Figure 3. Scatchard plot of effect of 10 nM T3 on [¹²⁵I]-PTH binding.

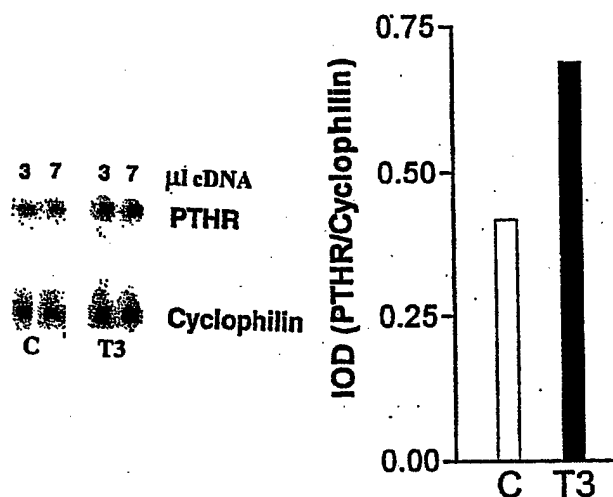


Figure 4. Effect of T3 on PTH receptor mRNA

There were no effects of T3 on cell number. In addition to potentiating the calcium signals elicited by PTH, T3 pretreatment enhanced the ratio of phosphorylated CREB (pCREB) to CREB (Figure 5), and amplified the inhibitory effect of PTH on alkaline phosphatase in the ROS 17/2.8 cells (Figure 6). Some of the data were reported in an abstract (12) and a manuscript is in press in Endocrinology.

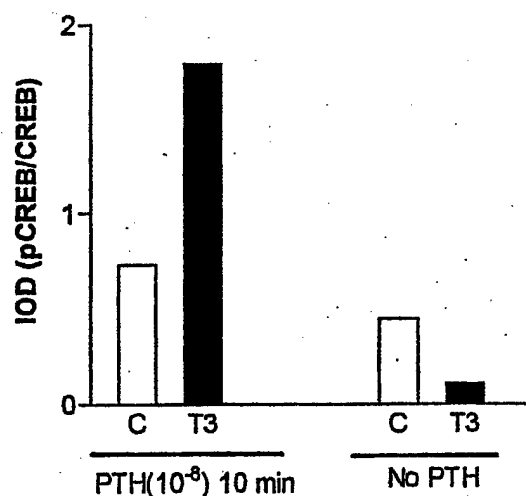


Figure 5. T3 pretreatment increases the Effect of PTH on pCREB/CREB ratio in ROS 17/2.8 cells

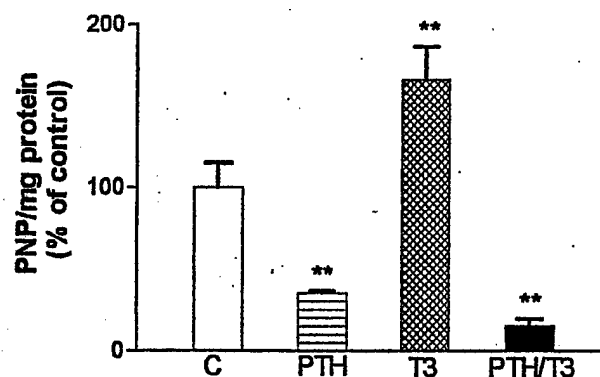


Figure 6. T3 pretreatment augments the effect of PTH to decrease alkaline phosphatase

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of the critical role of IGF-I in the anabolic effects of thyroid hormone on bone
- Demonstration that the effect of IGF-mediated anabolic effects of thyroid hormone are mediated through increased IGF-I production, rather than through an effect on IGF-I binding, since IGF-I binding was decreased by thyroid hormone
- Demonstration that transcriptional effects may contribute to the effect of thyroid hormone to increase IGF-I expression
- Demonstration that IL-6 is important in the effect of thyroid hormone to increase bone resorption
- Demonstration that thyroid hormone can augment IL-1 effects on IL-6 promoter expression, when a construct that represents the NF- κ B response element is the dominant regulatory element present
- Demonstration that in addition to interacting with IL-1, thyroid hormone can interact with PTH and increase the expression of the PTH receptor, with consequent downstream effects on signaling

REPORTABLE OUTCOMES

Manuscripts:

Huang BK, Golden LA, Tarjan G, Madison LD, Stern PH Insulin-Like Growth Factor Production is Essential for Anabolic Effects of Thyroid Hormone in Osteoblasts. J Bone Miner Res, 15:188-197, 2000

Gu, W-X., Stern, P.H., Madison, L.D., Du, G-G. Mutual up-regulation of thyroid hormone and parathyroid hormone receptors in osteoblastic ROS 17/2.8 cells. Endocrinology, in press.

Abstract (Oral Presentation):

Stern, P.H., Huang, B.K., Du, G-g., Golden, L., Madison, L. Mechanisms of thyroid hormone action on bone. International Conference on Progress in Bone and Mineral Research, 1998.

Abstracts (Posters):

Huang, B.K., Golden, L.A., Stern, P.H. Insulin-like growth factor as a possible mediator of thyroid hormone action in osteoblasts. International Conference on Progress in Bone and Mineral Research, 1998.

Huang, B., Golden, L.A., Madison, L.D., Stern, P.H. Insulin-like growth factor-I (IGF-I) mediates thyroid hormone anabolic actions in osteoblasts. ASBMR-IBMS Second Joint Meeting, 1998.

Gu, W., Stern, P.H., Du, G. Up-regulation by parathyroid hormone of thyroid hormone receptor binding in osteoblast-like ROS 17/2.8 cells. ASBMR-IBMS Second Joint Meeting, 1998.

Training:

Bill K. Huang carried out work demonstrating the role of IGF-I in the anabolic effects of thyroid hormone in osteoblasts as a Howard Hughes Medical Student Fellow.

CONCLUSIONS

The studies support the hypotheses that the anabolic and catabolic effects of T3 on bone require local cytokines and growth factors. Specifically, our findings indicate that IGF-I is critical for the anabolic effects of thyroid hormones to increase osteoblast proliferation and to stimulate the differentiated activities of the osteoblasts, and that IL-6 is important in the actions of T3 to stimulate bone resorption. Future studies will determine whether these are the only local factors required for these actions of T3. The catabolic effects were shown to require a basal effect of IL-1, which was then amplified by T3. At the time the studies were proposed, much less was understood about the mechanisms of osteoclastogenesis and osteolysis than are known now. We have begun to investigate the effects of T3 on ODF (RANKL/OPGL/TRANSC) expression in osteoblasts and in future studies will determine whether T3 can enhance the effects of other agents on ODF. Also, future studies will examine the role of local factors in T3 actions *in vivo*. In the studies on the mechanism of the effects of T3, evidence has been obtained supporting a hypothesis that the effects of thyroid hormone to increase the production of IGF-I and IL-1 is mediated at the level of mRNA expression. Further ongoing studies will further examine the

mechanism of these effects. It will be particularly germane to determine whether physiological changes, including changes with age, result in changes in the relative production of the of the local factors or of the sensitivity of bone to the local factors. Such findings could help to explain the differences in the effects of T3 during growth and during maturity and aging.

In summary, the significance of the work is that it has revealed pathways by which T3 can have either beneficial or deleterious effects on bone, these being the involvement of IGF-I in the anabolic effects and of IL-6 in the resorptive actions. Further, the studies have provided evidence for the mechanisms of the effects of T3 on the expression of these local bone factors. These findings help us to understand how disorders of the thyroid and supplemental thyroid hormones can influence bone integrity. They may provide new insights into sites at which therapy of bone disorders can be targeted.

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13. Gu, W-X., Stern, P.H., Madison, L.D., Du, G-G. Mutual up-regulation of thyroid hormone and parathyroid hormone receptors in osteoblastic ROS 17/2.8 cells. Endocrinology, in press. (Jan. 2001)

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APPENDICES

Abstracts:

Stern, P.H., Huang, B.K., Du, G-g., Golden, L., Madison, L. Mechanisms of thyroid hormone action on bone. International Conference on Progress in Bone and Mineral Research, 1998, Bone 22:421, 1998.

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Gu, W-X., Stern, P.H., Madison, L.D., Du, G-G. Mutual up-regulation of thyroid hormone and parathyroid hormone receptors in osteoblastic ROS 17/2.8 cells. Endocrinology, in press, (Jan. 2001).

MECHANISMS OF THYROID HORMONE ACTION ON BONE

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Thyroid hormone plays an important role in the normal development and mineralization of the skeleton. Thyroid hormone excess, especially in the adult, can result in loss of bone and predisposition to fractures. Since thyroid hormone regulates expression of multiple genes, many factors could potentially contribute to or modulate the effects of thyroid hormone on bone. Recent studies from our laboratory and other groups provide evidence to support the possible involvement of several diverse mechanisms. Evidence for three mechanisms that could play a role in the effects of thyroid hormone on bone will be presented: 1) Thyroid hormone increases the production of insulin-like growth factor-I in bone organ cultures and osteoblastic cells. This could contribute to anabolic effects of thyroid hormone. In support of this possibility, we find that interference with the action of insulin-like growth factor-I by antibody or antisense oligonucleotides can attenuate anabolic actions of thyroid hormone on osteoblastic cells. 2) Thyroid hormone potentiates the production of interleukin-1-mediated interleukin-6 production in bone organ cultures. The increase in interleukin-6, an osteoclastogenic factor, could contribute to the catabolic effects of thyroid hormone. In support of this possibility, we find that an antibody to the interleukin-6 receptor can decrease the resorptive effects of thyroid hormone in bone organ culture. 3) Thyroid hormone stimulates the expression of receptors to parathyroid hormone in osteoblastic cells. This could conceivably contribute to either the anabolic or catabolic effects of thyroid hormone by influencing the response to circulating parathyroid hormone and/or locally released parathyroid hormone-related peptide. Factors that influence these processes, including age-related changes in their sensitivity and physiological or exogenous antagonists or promoting factors, could determine the ultimate effect of thyroid hormone on bone in an individual.

Bone 22: 421, 1998

INSULIN-LIKE GROWTH FACTOR I (IGF-I) AS A POSSIBLE MEDIATOR OF THYROID HORMONE ACTION IN OSTEOBLASTS.

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Thyroid hormone (T3) is a critical regulator of skeletal function, with both anabolic and catabolic effects. Recent studies, including those from our laboratory, suggest that T3 increases the production of IGF-I and the expression of IGF-I mRNA in osteoblasts. IGF-I is an important endogenous regulatory factor in bone with autocrine/paracrine effects. To assess the potential role of IGF-I as a mediator of T3 effects, we characterized phenotypic markers of osteoblast activity in MC3T3-E1 cells and in primary mouse osteoblasts exposed to T3 alone and in the presence of agents that interfere with IGF-I actions. 72 hour treatment with 10 nM T3 significantly increased ³H-proline incorporation, alkaline phosphatase (ALP), and osteocalcin (OCN). However, the same treatment caused a significant decrease in ³H-thymidine uptake. When the cells were simultaneously treated with α IR3, a neutralizing monoclonal antibody to IGF-I receptor, the stimulatory effects of T3 were attenuated. Similar results were also observed when the cells were incubated with phosphothiolated antisense oligonucleotide (AS) directed against the IGF-I receptor gene prior to treatment with T3. Treatments with either α IR3 or AS alone resulted in measurements similar to the corresponding control. Results from experiments with MC3T3-E1 cells and primary osteoblasts are summarized below as % of control \pm SD.

	Treatments			
	control	10 nM T3	10 nM T3 + α IR3	10 nM T3 + AS
³ H-proline incorporation ¹	100	143 \pm 4*	116 \pm 7	NA ²
ALP ³	100	165 \pm 30**	NA	90 \pm 10 [†]
OCN ³	100	627 \pm 69**	NA	308 \pm 39 [†]
³ H-thymidine incorporation ¹	100	60 \pm 3**	72 \pm 7	NA

¹ MC3T3-E1 cells; ² Primary osteoblasts; ³ NA - not available

* p<0.01 vs. control; ** p<0.001 vs. control; [†] p<0.01 vs. 10 nM T3

Our data show 10 nM T3 to have anabolic effects on osteoblasts. T3 seems to promote the cell phenotype toward differentiated osteoblastic functions rather than toward proliferation. IGF-I appears to play an important role in skeletal effects of T3 and may be one of the mediators of T3 action on bone.

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SA142

Insulin-Like Growth Factor-I (IGF-I) Mediates Thyroid Hormone Anabolic Actions in Osteoblasts. Bill K. Huang,^{*1} Laurence A. Golden,^{*1} Laird D. Madison,^{*2} Paula H. Stern,¹ ¹Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, IL, ²Endocrinology, Northwestern University Medical School, Chicago, IL.

Thyroid hormone (T3) is a critical regulator of skeletal function, having both anabolic and catabolic effects. Recent studies, including those from our laboratory, indicate that T3 increases the production of insulin-like growth factor-I (IGF-I) and the expression of IGF-I mRNA in osteoblasts. IGF-I is an important endogenous regulatory factor in bone with autocrine/paracrine effects. To assess the potential role of IGF-I as a mediator of T3 actions, we characterized phenotypic markers of osteoblast activity in MC3T3-E1 osteoblastic-like cells and in primary mouse osteoblasts exposed to T3 alone and in the presence of agents that interfere with IGF-I actions. The markers measured included (1) ³H-proline incorporation as a measurement of protein synthesis, (2) alkaline phosphatase activity, and (3) osteocalcin production. We also measured ³H-thymidine uptake to assess proliferative activity. Cells treated with 10 nM T3 for 72 hours had significantly increased ³H-proline incorporation, alkaline phosphatase, and osteocalcin. When the cells were simultaneously treated with α IR3, a neutralizing monoclonal antibody to IGF-I receptor, or JB1, an IGF-I analogue antagonist, the stimulatory effects of T3 were attenuated. Similar results were also observed when the cells were transfected with a phosphothiolated antisense oligonucleotide complementary to the IGF-I receptor gene, prior to treatment with T3. Cells transfected with nonsense oligonucleotides did not show attenuated responses. Treatment with α IR3, JB1, or antisense oligonucleotide alone did not have significant effects. IGF-I alone did not stimulate phenotypic markers of osteoblast activity. However, both IGF-I and T3 produced time-dependent, biphasic effects on mitogenic activity, and the stimulatory effects of T3 on proliferative activity were blocked by antagonists to the IGF-I receptor. In summary, we have shown that anabolic effects of T3 on ³H-proline incorporation, alkaline phosphatase activity, and osteocalcin are attenuated by treatments that interfere with IGF-I actions, indicating that IGF-I plays an important skeletal anabolic effects of T3. Since IGF-I alone failed to stimulate these differentiated functions under the conditions where T3 elicited responses, our results suggest that IGF-I production is necessary but not sufficient for the anabolic effects of T3.

Bone 23: S561, 1998

W221

Up-regulation by Parathyroid Hormone of Thyroid Hormone Receptor Binding in Osteoblast-like ROS17/2.8 Cells. Wen-Xia Gu,² Paula H. Stern,¹ Guo-Guang Du,¹ ¹Molecular Pharmacology & Biological Chemistry, Northwestern University, Chicago, IL, ²Center for Endocrinology, Metabolism & Molecular Medicine, Northwestern University, Chicago, IL.

Parathyroid hormone (PTH) and thyroid hormone (T3) both play important roles in bone remodeling. There may be interaction between T3 and PTH; results from two patients with concomitant Graves' disease and primary hyperparathyroidism suggested that T3 potentiated the osteoclastic effect of PTH (Arem et al. 1986). We have previously found that PTH receptor binding and PTH receptor mRNA expression were increased following T3/T4 treatment in ROS 17/2.8 cells. In the present study we examined the effect of PTH on thyroid hormone receptor (TR) binding in osteoblast-like ROS 17/2.8 cells. Confluent cells were serum-depleted for 24 h. Cells were supplemented with 0.1% FBS and treated with PTH (10⁻¹¹ - 10⁻⁷ M) or PTHrP (10⁻⁹ M) for 3-4 days. Medium was changed every day. Nuclear extracts were prepared and the protein contents were standardized. Extracts were incubated overnight at 4°C with [¹²⁵I]-lithyronine (0.25nM). Free and bound [¹²⁵I] were separated by vacuum filtration through membranes (Millipore HA.0.45µm), and the radioactivity on the membrane was measured. Specific binding was calculated by subtraction of the non-specific binding (binding in the presence of non-labeled T3, 10⁻⁷ M). The results showed that both PTH and PTHrP stimulated TR binding with maximum effects of PTH observed at 10⁻⁹ - 10⁻⁸ M. The specific binding gradually increased over 4 days of PTH treatment. These findings indicate that TR can be up-regulated by PTH. Taken together with our previous findings that T3 can up-regulate PTH receptors, it appears that the effects of PTH and T3 can be strengthened by mutual up-regulation of their receptors.

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Insulin-Like Growth Factor I Production Is Essential for Anabolic Effects of Thyroid Hormone in Osteoblasts*

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ABSTRACT

Thyroid hormone (T3) and insulin-like growth factor I (IGF-I) are critical regulators of skeletal function. T3 increases IGF-I production in bone. To assess the potential role of IGF-I as a mediator of T3 actions, we characterized phenotypic markers of osteoblast activity in two osteoblast models, normal mouse osteoblasts and MC3T3-E1 cells, exposed to T3 alone or under conditions that interfere with IGF-I actions. T3 significantly increased osteoblast ³H-proline incorporation, alkaline phosphatase (ALP), and osteocalcin. Both α IR3, a neutralizing monoclonal antibody to the IGF-I receptor, and JB1, an IGF-I analogue antagonist, attenuated the stimulatory effects of T3. T3 effects also were decreased in cells transfected with antisense oligonucleotide (AS-ODN) to the IGF-I receptor gene. Both IGF-I and T3 had mitogenic effects that were inhibited by the antagonists. IGF-I by itself did not stimulate ³H-proline incorporation, ALP, and osteocalcin in the models used, revealing that although IGF-I is essential for the anabolic effects of T3, it acts in concert with other factors to elicit these phenotypic responses. (J Bone Miner Res 2000;15:188–197)

Key words: insulin-like growth factor I, thyroid hormone, bone formation markers, growth factor regulation, osteoblasts

INTRODUCTION

THYROID HORMONES (T3/T4) are critical regulators of skeletal development and maturation. Evidence demonstrating effects of T3 to promote both the formation and breakdown of bone can be found in clinical observations as well as in vivo investigations in animals.^(1,2) Long-standing juvenile hypothyroidism can cause severe growth retardation when thyroid hormones are not replaced.⁽³⁾ T4 treatment in children with congenital hypothyroidism resulted in a positive correlation between the bone age and the dose of T4 administered.⁽⁴⁾ Although in children and in young animals excess T3 causes enhanced bone growth,^(5,6) it leads to bone

loss in adults.^(7,8) It is well established that both bone formation and resorption are markedly increased in hyperthyroidism.⁽⁹⁾ Greater increases in the resorption markers than the formation markers suggest an imbalance between resorption and formation, leading to a net loss of cortical and trabecular bone volume.⁽¹⁰⁾

Despite the clinical importance of the regulatory actions of T3 on bone, little is known about the mechanisms by which this occurs. Recent studies have identified the presence of thyroid hormone receptors in osteoblast-like cells and immortalized osteosarcoma cells and have further demonstrated that these receptors are functional.^(11,12) The expression of certain thyroid hormone receptor isoforms has been shown in osteoclasts.⁽¹³⁾ The expression of many genes in a variety of tissues, including bone, is regulated by T3.⁽¹⁴⁾ Specifically, production of several osteoblast phenotypic markers such as alkaline phosphatase (ALP), osteocalcin, and collagen are affected by T3 in a number of osteoblastic cells.^(15,16) Furthermore, there is evidence suggesting that

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certain effects of T3 on bone remodeling are mediated through the production of local factors. Factors such as prostaglandins and interleukin-1 (IL-1) have been implicated as mediators of the resorptive effects of T3 on bone.^(17,18)

As one of the most abundant growth factors present in bone, insulin-like growth factor I (IGF-I) represents a likely mediator of the anabolic actions of systemic hormones. It is found in the plasma and also is produced in many tissues, where it has both autocrine and paracrine action.^(19,20) IGF-I stimulates bone production by increasing osteoblast proliferation and matrix synthesis.^(21,22) T3 causes an increase in IGF-I production in UMR-106 osteoblastic cells and bone organ cultures.^(23,24) IGF-I messenger RNA (mRNA) in MC3T3-E1 osteoblastic cells also is increased after T3 treatment.⁽²⁵⁾ The IGF-I gene is regulated by several different hormones. Growth hormone (GH) is the prototypic regulator of the IGF-I gene, especially in the liver. T3 has been shown to interact with GH and, together, these hormones regulate IGF-I production by the liver and other tissues.⁽²⁶⁾ Levels of GH and IGF-I are reduced in hypothyroidism, but treatment with GH alone failed to restore normal growth in hypothyroid children.⁽²⁷⁾ Although the biological significance is still unclear, estrogen can induce IGF-I mRNA in osteoblast cell lines.⁽²⁸⁾ In addition, parathyroid hormone (PTH) also increases IGF-I mRNA in bone cell cultures.⁽²⁹⁾ IGF-I appears to mediate specific anabolic effects of PTH on bone; interference with IGF-I blocks the anabolic actions of PTH.⁽²²⁾

The purpose of this study was to determine whether IGF-I activity is important for the skeletal effects of T3 on bone formation. Because both IGF-I and T3 have anabolic actions on bone and T3 also increases IGF-I production in bone, it was important to determine whether IGF-I could be a mediator of T3 actions on bone. We used several different complementary approaches to interfere specifically with the IGF-I actions in osteoblasts and to determine whether these interventions would prevent the anabolic effects of T3. Osteoblastic functions assessed included total protein synthesis based on proline incorporation and the production of the phenotypic markers of osteoblastic activity, osteocalcin, and ALP. We also measured the mitogenic activity of the osteoblasts. Normal mouse osteoblasts and/or MC3T3-E1 cells, a widely used clonal osteoblast cell line, were used for the studies.

Our data show that interference with IGF-I actions attenuates T3-stimulated osteoblastic functions and thus support a model in which IGF-I represents a critical factor for the anabolic effects of T3.

METHODS

Cell culture

Primary osteoblast cultures were prepared from calvarial bones obtained from 6–7-day-old neonatal mice.⁽³⁰⁾ Mice were housed and killed in accordance with policy at the Northwestern University Animal Care and Use Facility. After the calvarial bones were dissected, cells were released from the bone by six sequential 20-minute collagenase

digestions. A pool from digestions 2–6 was collected and seeded into 75-cm² flasks for culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 100 µg/ml penicillin and streptomycin. This primary culture was allowed to grow to confluence and was trypsinized for subculture onto appropriate experiment dishes (either 24- or 96-well multi-well plates). This subculture (technically passage 2) expressed a consistent osteoblastic phenotype based on ALP, osteocalcin (OCN), and ³H-proline incorporation, and it was used to study osteoblast responses to agonist stimulation.

Mouse osteoblastic MC3T3-E1 cells were cultured in α modified essential medium (α -MEM) supplemented with 5% FBS and 50 µg/ml gentamycin. Cells were passaged every 7 days by harvesting with 0.1% trypsin-EDTA and reseeded in 75-cm² flasks. For experiments, MC3T3-E1 cells between passages 3 and 14 were seeded into 60-mm dishes or 24- or 96-well cluster dishes. Assessment of mature osteoblastic phenotypic markers such as ALP and OCN in MC3T3-E1 cell was performed in cells after 18 days of culture time. The long culture time was required for MC3T3-E1 cells to develop into more mature osteoblasts with the ability to express detectable levels of ALP.⁽³¹⁾ The cells were seeded and grown in normal serum media for 3 days and the cells then were cultured in T3-free medium for 18-days before treatment with agonists. Medium was changed every 3 days.

For experiments assessing mitogenic activity, once the cells reached 60–80% confluence after seeding, cells were made quiescent by incubation for 18 h in medium supplemented with 0.1% bovine serum albumin (BSA) and antibiotic (hereafter designated as serum-free medium). For experiments involving effects of hormonal manipulation on osteoblast functions, cells were cultured in hormone-depleted medium (designated as T3-free medium) after the cells reached confluence in normal medium. Treatment with Dowex AF-1-X-10 resin (Sigma, St. Louis, MO, USA) was used to remove T3 and T4 hormones from serum with reasonable selectivity. This method, originally reported by Samuels et al., reduced T3 from 150 ng/dl to <2.0 ng/dl and T4 from 6.8 µg/dl to 0.08 µg/dl in calf serum.⁽³²⁾ T3-free medium contained 5% resin-treated FBS, 50 µg/ml ascorbic acid, and appropriate antibiotics.

[³H]Thymidine incorporation

Osteoblasts were plated in 24-well cluster dishes with normal medium at 20,000 cells/well. They were made quiescent as described above and then cultured in serum-free medium with either T3 or IGF-I for a predetermined treatment period. The concentrations of T3 or IGF-I used were the lowest concentrations that caused a significant response and they were determined based on preliminary studies. [³H]Thymidine (0.5 µCi/ml) was added to the cells during the last hour of the treatment in the continued presence or absence of the agonists. At the termination of treatment, [³H]thymidine-containing medium was removed. The cells were washed once with ice-cold phosphate-buffered saline (PBS), once with 10% trichloroacetic acid (TCA), incubated on ice for 15 minutes with 10% TCA, and

after one wash with ice-cold 95% ethanol, radioactivity was extracted with 1N NaOH for assay by liquid scintillation.

[³H]Proline incorporation

Osteoblasts were subcultured onto 24-well cluster dishes with normal medium at 35,000 cells/well. After the culture reached confluence, cells were hormone-depleted by culturing in T3-free medium for 48 h before treatment. Cells were treated for 72 h unless otherwise indicated. For measurement of proline incorporation, cells were incubated for the last 2 h of the treatment period with [³H]proline (1 μ Ci/ml). Samples were prepared similarly to the procedures described for [³H]thymidine incorporation. Incorporation of the label into the TCA-precipitable fraction was measured by liquid scintillation counting.

OCN

Osteoblasts were seeded onto 96-well cluster dishes at 16,000 cells/well in normal medium. Confluent cells then were cultured in T3-free medium before agonist stimulation of predetermined length. Media from the final 72 h of treatment were collected and stored at -20°C until assay for OCN. OCN secreted by the osteoblasts was measured by a commercial radioimmunoassay (RIA; Biomedical Technologies, Stoughton, MA, USA). This assay uses goat anti-mouse OCN and mouse ¹²⁵I-OCN tracer. This assay is specific for mouse OCN and recognizes total OCN. The limit of detection of the assay is 1.56 ng/ml.

ALP activity

Osteoblasts were seeded in 96-well culture cluster dishes at 16,000 cells/well in normal medium. Confluent monolayer cells then were cultured in T3-free medium before agonist stimulation. Cells were treated with agonists for 72 h before ALP measurement. ALP in cell lysates was measured by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate using a protocol modified from Schlossman et al.⁽³³⁾ Briefly, medium was removed from the treated cells and the monolayers were washed with cold PBS. After the PBS was aspirated, 100 μ l diethanolamine (50 mM, pH 10.5) was added to disrupt the monolayer and 50 μ l of 2.5 mM *p*-nitrophenyl phosphate in glycine buffer (100 mM glycine, 2 mM MgCl_2) was added as substrate. The entire complex was incubated at 37°C for 30 minutes, and the reaction was stopped by the addition of 0.1N NaOH. Absorbance was read on a Dynatech (Chantilly, VA, USA) MR5000 spectrophotometer at 410 nm. Activity was calculated in reference to a standard curve of *p*-nitrophenol. To assess the effect of IGF-I receptor down-regulation on either T3- or IGF-I-stimulated ALP activity, MC3T3-E1 cells precultured in T3-free medium for 18 days were transfected with 1.66 ng/well of either antisense oligonucleotide (AS-ODN) or mismatch oligonucleotide (MS-ODN) for 4 h using the protocol described below. After the transfection period, cells were returned to T3-free medium and subsequently exposed to either IGF-I or T3 for 72 h. ALP activity was measured at the end of the treatment period.

Antagonists to IGF-I receptor

To determine the effects of IGF-I receptor antagonists on IGF-I- or T3-induced cellular functions, experiments were performed in which the normal mouse osteoblasts or MC3T3-E1 cells were exposed to the agonists alone or in the presence of antagonists. α IR3 (Calbiochem, San Diego, CA, USA), a monoclonal antibody specific against the IGF-I receptor and JB1 (Peninsula Laboratories, Inc., San Carlos, CA, USA), a peptide analogue of IGF-I, were used as antagonists. Both agents have specificity for the IGF-I receptor and neutralizing properties that block the IGF-I signaling pathway.^(34,35) A mouse myeloma nonspecific immunoglobulin G (IgG) antibody also was used to confirm specificity in the osteoblast model.

Antisense IGF-I receptor oligonucleotides

The AS-ODN, 5'-CCC TCC TCC GGA GCC-3', is complementary to a sequence around the AUG-site of the mouse IGF-I receptor mRNA sequence; MS-ODN is a scrambled version of AS-ODN, 5'-CCC GGA TCC TCC GCC-3'. Dr. Sergei Gryaznov (personal communication, 1997) kindly provided these sequences. To assess the efficacy of the AS-ODN, confluent MC3T3-E1 osteoblasts seeded in 60-mm dishes were transfected with either AS-ODN or MS-ODN (3.22 μ g DNA/dish) using Lipofectamine-PLUS reagent following the manufacturer's protocol (Gibco, Grand Island, NY, USA). The cells were transfected in serum-free medium for 4 h and the medium then was replaced with normal growth medium. Levels of IGF-I receptor in transfected cells were assessed 48–72 h posttransfection.

Western immunoblot

IGF-I receptor expression was determined by Western immunoblotting using methods described previously.⁽³⁰⁾ Briefly, an aliquot of the whole cell lysates of MC3T3-E1 cells was used for determination of protein using the method of Lowry.⁽³⁶⁾ Fifteen micrograms of protein was applied per lane, and electrophoresis was performed under denaturing conditions on a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel. After overnight wet transfer to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA), the blot was probed with a rabbit IGF-I receptor β -subunit antibody (Santa Cruz, Santa Cruz, CA, USA) at a 1:500 dilution and followed by subsequent incubation with a goat anti-rabbit, peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO, USA) at a 1:2000 dilution. The blot was visualized by enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ, USA) using Kodak X-OMAT LS film. For normalization purposes, the same blot also was probed with a rabbit β -actin antibody (Sigma, St. Louis, MO, USA). Immune complexes were quantified by densitometry using a Bio-Rad imaging densitometer (BioRad, Hercules, CA, USA).

Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was carried out by one-way analysis of variance. Signifi-

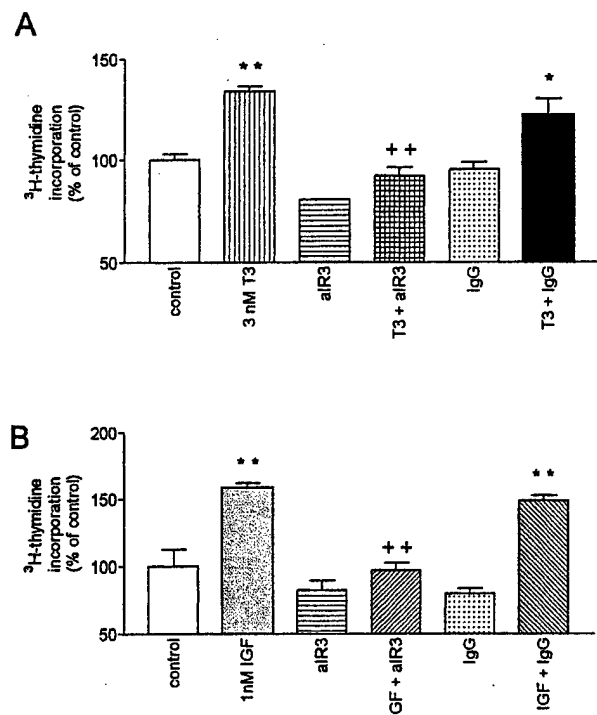


FIG. 1. IGF-I and T3 stimulate mitogenic activity in normal mouse osteoblasts and antibody to IGF-I receptor (α IR3) blocks the mitogenic effects of IGF-I and T3. (A) Quiescent normal osteoblasts were treated with 3 nM T3 with or without antibody (1.5 μ g/ml α IR3 or 1.5 μ g/ml control IgG) alone for 12 h or in the presence of both antibody and agonist. **Significant difference from control, $p < 0.01$. *Significant difference from control, $p < 0.05$. ++Significant difference from 3 nM T3, $p < 0.01$. (B) Quiescent normal mouse osteoblasts were treated with 1 nM IGF-I with and without antibody (1.5 μ g/ml α IR3 or 1.5 μ g/ml control IgG) for 12 h. **Significant difference from control, $p < 0.01$. ++Significant difference from 1 nM IGF-I, $p < 0.01$. [³H]Thymidine incorporation reflects value from per well and is expressed as percent control. Data represent mean \pm SEM, $n = 3$ or 4 for each treatment group from a single experiment. Experiments were repeated at least three times; each graph is from one representative experiment.

cance of differences among means was determined by post hoc testing, using Tukey's method.

RESULTS

Effects of blockade of IGF-I receptors on mitogenic activity of T3 and IGF-I in osteoblasts

Three nM T3 increased [³H]thymidine incorporation in quiescent normal mouse osteoblasts (Fig. 1A). This result also was observed with MC3T3-E1 cells treated with 10 nM T3 (Fig. 2A). As shown, 12-h T3 treatment increased [³H]thymidine incorporation in normal mouse osteoblasts and MC3T3-E1 cells by 33% and 19%, respectively. Treatment with IGF-I significantly increased [³H]thymidine incorporation in both cell models. A 59% increase in [³H]thymi-

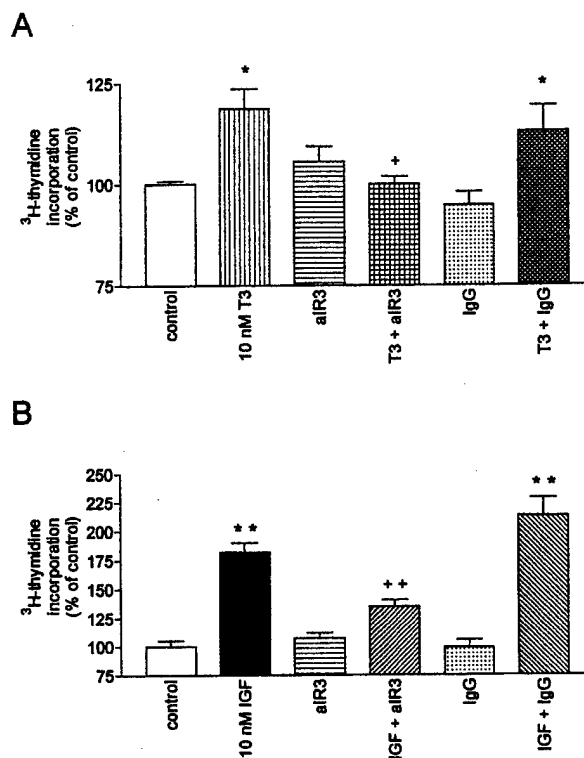


FIG. 2. Antibody to IGF-I receptor (α IR3) blocks the mitogenic effects of T3 and IGF-I in MC3T3-E1 cells. (A) Quiescent MC3T3-E1 cells were treated with 10 nM T3 with or without antibody (1.5 μ g/ml α IR3 or 1.5 μ g/ml control IgG) for 12 h. *Significant difference from control, $p < 0.05$. +Significant difference from 10 nM T3, $p < 0.05$. (B) Quiescent MC3T3-E1 cells were treated with 10 nM IGF-I with or without antibody (1.5 μ g/ml α IR3 or 1.5 μ g/ml control IgG) for 12 h. **Significant difference from control, $p < 0.01$. ++Significant difference from 10 nM IGF-I, $p < 0.01$. [³H]Thymidine incorporation per well is expressed as percent control. Data represent mean \pm SEM, $n = 3$ or 4 for each treatment group from a single experiment. Experiments were repeated at least three times; each graph is from one representative experiment.

dine incorporation was seen in normal osteoblasts treated with 1 nM IGF-I for 12 h (Fig. 1B). MC3T3-E1 cells treated with 10 nM IGF-I for 12 h showed an 82% increase compared with control cells (Fig. 2B). The concentrations of T3 and IGF-I used were the lowest levels that showed a significant effect during preliminary studies.

When normal mouse osteoblasts or MC3T3-E1 cells were coincubated with T3 and a neutralizing antibody to the IGF-I receptor (α IR3), the stimulatory effect of T3 on [³H]thymidine incorporation was blocked (Figs. 1A and 2A). Treatment with T3 in the presence of 1.5 μ g/ml of α IR3 attenuated the stimulatory effect of T3 to the level of nonstimulated control cells. The same concentration of α IR3 also blocked the increased [³H]thymidine incorporation stimulated by IGF-I in both cell models (Figs. 1B and 2B). Treatment with α IR3 alone had no effect on basal proliferative responses. A nonspecific, control IgG antibody failed to

inhibit either IGF-I or T3-stimulated responses in MC3T3-E1 cells or normal mouse osteoblasts.

Effects of IGF-I receptor antagonists on T3-stimulated osteoblastic functions

[³H]proline incorporation: The effects of 72h T3 treatment on ³H-proline incorporation in confluent normal mouse osteoblasts precultured in T3-free medium are illustrated in Fig. 3A. Ten-nM T3 caused a 41% increase in osteoblast [³H]proline incorporation. In this model and under the conditions used, 10 nM IGF-I elicited no stimulation. There was no synergism in the observed responses when the cells were treated with both T3 and IGF-I (data not shown). When cells were treated with 10 nM T3 and in the presence of either α IR3 or peptide analogue of IGF-I, JB1, the T3-stimulated [³H]proline incorporation was attenuated to the level of control. Although both α IR3 and JB1 appear to have slight stimulatory effects on basal [³H]proline incorporation, these changes were not statistically significant.

The 10 nM T3 also stimulated [³H]proline incorporation in confluent MC3T3-E1 cells grown in T3-free medium. As shown in Fig. 3B, 10 nM T3 caused a 43% increase in [³H]proline incorporation. As in normal mouse osteoblasts, no significant stimulation was observed with 10 nM IGF-I treatment. The stimulatory effect of T3 on [³H]proline incorporation was attenuated by α IR3. The α IR3 alone did not change ³H-proline incorporation when compared with control.

OCN: For these studies, normal mouse osteoblasts, grown to confluence and precultured in T3-free medium for 48 h, were incubated with 1 nM T3 for 72 h. Higher concentrations of T3 did not elicit greater stimulation and we chose the lowest concentration that elicited a significant response for the antagonist studies. The culture media were collected and assayed for OCN production using an RIA. In media from control cells, OCN was 2.3 ± 2.6 ng/ml (mean \pm SEM, $n = 6$). As shown in Fig. 4A, 1 nM T3 induced an approximately 50-fold increase in OCN production by the osteoblasts. Similar to the findings on ³H-proline incorporation, 10 nM IGF-I did not stimulate detectable OCN production under the conditions used. To determine whether IGF-I was required for the T3-stimulated OCN production, the cells were treated with T3 in the presence of the IGF-I receptor antibody, α IR3. The α IR3 (0.75–3.0 μ g/ml) significantly decreased T3-stimulated OCN production. The α IR3 alone had no effects on OCN production.

T3 also stimulated OCN production in MC3T3-E1 cells. As shown in Fig. 4B, the maximal effect was obtained with 10 nM T3. OCN from control MC3T3-E1 cells was 0.72 ± 1.5 ng/ml (extrapolated value, below the detection limit of the assay) and 10 nM T3 stimulated OCN production to 11 ± 5 ng/ml. Although OCN stimulation in MC3T3-E1 cells treated with T3 was statistically significant compared with that in the control cells, the OCN concentration in MC3T3-E1 cells was significantly less than levels observed in normal osteoblasts. For these reasons, we focused our efforts on the normal osteoblasts and did not study the effects of IGF-I antagonists on T3-stimulated OCN production in the MC3T3-E1 cells. As was found in normal mouse osteo-

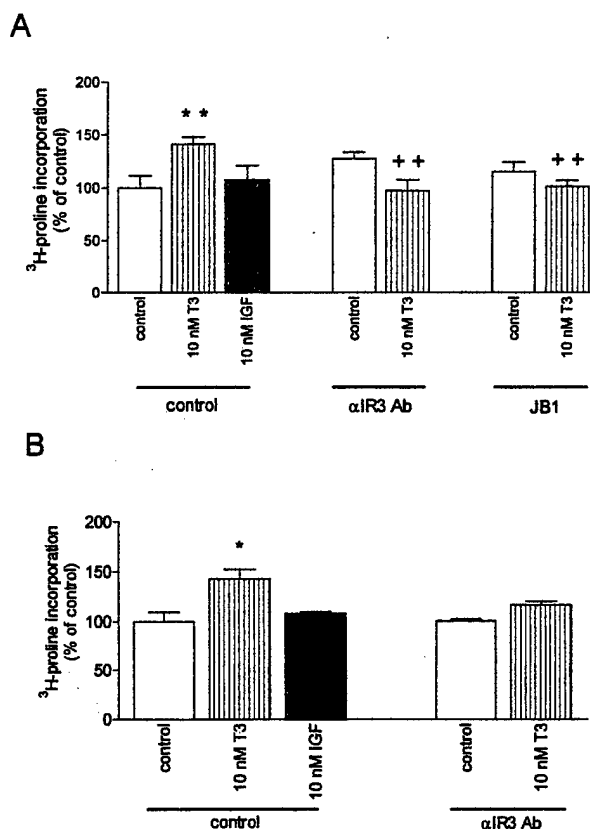


FIG. 3. Antagonists to IGF-I receptor inhibit T3 stimulate [³H]proline incorporation. (A) Confluent normal mouse osteoblasts were treated with agonists (10 nM T3 or 10 nM IGF-1) for 72 h with or without either α IR3 (1.5 μ g/ml) or JB1 (2 μ g/ml). (B) Confluent MC3T3-E1 cells were treated with agonists (10 nM T3 or 10 nM IGF-1) for 72 h with or without α IR3 (1.5 μ g/ml). **Significant difference from control, $p < 0.01$. ++Significant difference from 10 nM T3, $p < 0.01$. [³H]proline incorporation for each well is expressed as percent control. Data represent mean \pm SEM, $n = 4$ for each treatment group from single experiment. Experiments were repeated at least three times for each cell type; each graph is from one representative experiment.

blasts, IGF-I did not increase OCN production in the MC3T3-E1 cells.

ALP: We attempted to study the effects of T3 on ALP activity normal mouse osteoblasts. As shown in Fig. 5A, the expression of ALP in these cells did not show any stimulation after 72-h treatment with T3 or IGF-I. The baseline ALP level in confluent mouse osteoblasts was four times the baseline ALP level in confluent MC3T3-E1 cells.

A stimulatory effect of T3 on ALP in MC3T3-E1 cells was evident when confluent MC3T3-E1 cells grown for 18 days were treated with 10 nM T3 for 72 h (total culture time of 21 days). T3-treated cells showed a 34% increase in ALP activity compared with nontreated cells (Fig. 5B). The 10 nM IGF-I treatment did not change ALP levels significantly. Cotreatment with 10 nM IGF and 10 nM T3 did not produce stimulation significantly different from the response to 10 nM T3 alone (data not shown).

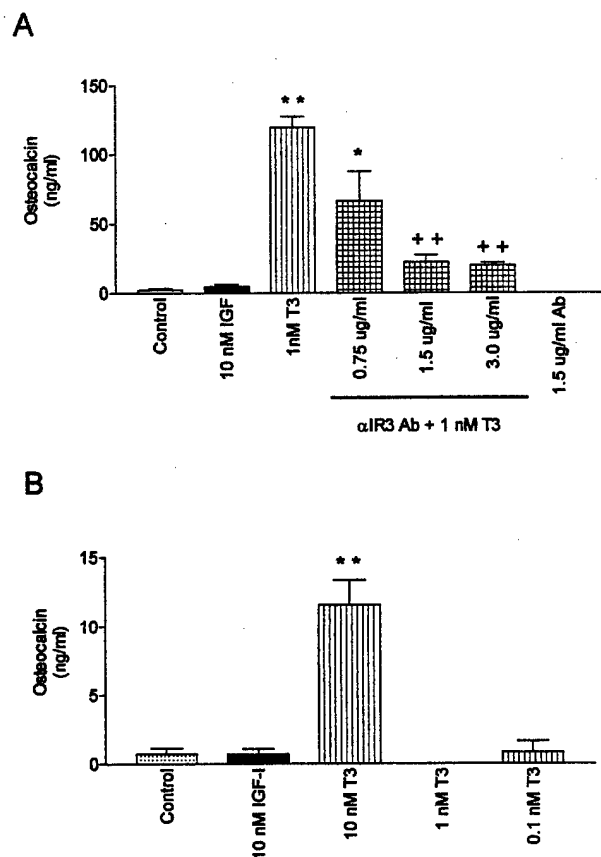


FIG. 4. T3 stimulates OCN production and α IR3 (Ab) blocks the effects of T3 on OCN. (A) Confluent normal osteoblasts were treated with agonists (1 nM T3 or 1 nM IGF-I) with or without α IR3 antibody (0.75, 1.5, or 3 μ g/ml.) (B) Confluent MC3T3-E1 cells were treated with T3 (0.1, 1.0, and 10 nM) or 10 nM IGF-I. OCN was measured in the collected conditioned medium and reflects the OCN concentration per well of confluent monolayer. Data represent mean \pm SEM, $n = 6-12$ for each treatment group. Data were gathered from at least three separate experiments and combined for analysis. **Significant difference from control, $p < 0.001$; *Significant difference from control, $p < 0.01$; ++Significant difference from 10 nM T3, $p < 0.01$.

The T3-stimulated ALP activity was attenuated in MC3T3-E1 transfected with an AS-ODN that is complementary to mouse IGF-I receptor mRNA (Fig. 5B). In these cells, T3 failed to elicit a significant increase in ALP. Cells transfected with 1.66 μ g/ml of the MS-ODNs did not cause an attenuated response to T3. Western immunoblotting for IGF-I receptor in MC3T3-E1 cells confirmed the specificity of AS-ODN for the IGF-I receptor. As shown in Fig. 6, MC3T3-E1 cells transfected with 1.66 μ g/ml AS-ODN showed a 40% decrease in IGF-I receptor expression. Cells transfected with same amount of MS-ODN did not show decreased receptor expression. Transfection of the MC3T3-E1 cells with either AS-ODN or MS-ODN had no effect on the levels of actin protein expression.

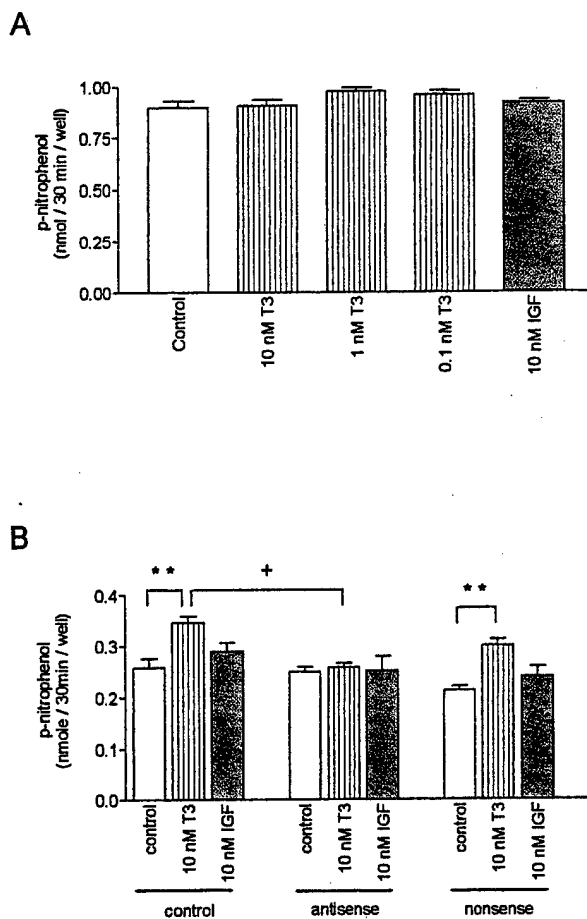


FIG. 5. Effects T3 treatment on osteoblast ALP activity. (A) Lack of significant stimulation of ALP activity by T3 (0.1, 1.0, and 10 nM) in normal osteoblasts. (B) T3 stimulates ALP activity in MC3T3-E1 cells, and IGF-I receptor AS-ODN blocks T3-induced ALP activity. MC3T3-E1 cells were transfected with either AS-ODN or MS-ODN and treated with 10 nM T3 or 10 nM IGF-I for 72 h. **Significant difference from the respective control, $p < 0.01$. +Significant difference from nontransfected cells treated with 10 nM T3, $p < 0.05$. ALP activity was measured per well of confluent osteoblast monolayer. Data represent mean \pm SEM, $n = 6-12$ for each treatment group from a single experiments. Experiments were repeated at least three times for each cell type; each graph is from one representative experiment.

DISCUSSION

The importance of T3 to maintain normal skeletal physiology is well accepted. However, the mechanism of T3 effects on bone remodeling remains to be elucidated. We focused our project specifically on studying the anabolic actions of T3 on bone. These anabolic responses included osteoblast proliferation and osteoblast phenotypic markers such as ALP activity, OCN production, and collagen synthesis. An improved understanding of this basic anabolic process elicited by T3 could be beneficial in the management of skeletal

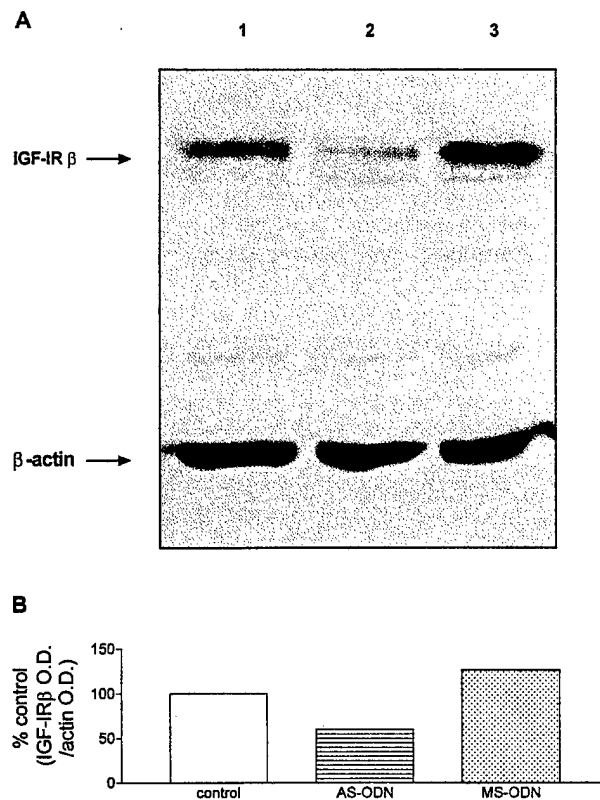


FIG. 6. IGF-I receptor AS-ODN decreases IGF-I receptor expression in MC3T3-E1 cells. The IGF-I receptor protein level was determined by Western immunoblotting with an anti-IGF-I receptor β -antibody. (A) Levels of IGF-I receptor expression in control (lane 1) MC3T3-E1 cells and in cells transfected with either 1.66 μ g/ml AS-ODN (lane 2) or MS-ODN (lane 3) were determined using Western immunoblotting. Levels of β -actin expression were measured and used for normalization. The experiments were repeated at least three times; presented graphs are representative of one experiment. (B) Expression was quantified by densitometry and expressed as the ratio of IGF-I receptor to actin. Data are presented as percent control.

problems in patients with altered thyroid status caused by either disease processes or of iatrogenic origins and is thus of clinical importance.

Results from previous studies that showed T3 stimulates IGF-I production in bone models suggest the possibility that IGF-I could be responsible for some of the anabolic effects of T3 in bone tissues. Both T3 and IGF-I have skeletal anabolic effects *in vivo*: hypothyroid patients, if untreated, often show a bone age delayed more than 2 standard deviations from their chronological age; and with subsequent thyroid replacement therapy, the bone age positively correlates with the concentration of serum T4.⁽⁴⁾ A patient with a T3 receptor mutation (homozygous deletion of threonine-337 in TR β gene) showed similar abnormal skeletal development.⁽³⁷⁾ Similarly, the importance of IGF-I to skeletal systems is evident from the delayed bone development in IGF-I and IGF-I gene knock-out mice; mice with IGF-I receptor mutations show significantly delayed or

arrested ossification in the developing skeleton.⁽³⁸⁾ These observations, taken together, further suggest the likelihood that IGF-I may be a candidate to mediate the stimulatory effects of T3 on bone formation or be essential for these stimulatory effects. We therefore tried to determine whether blocking IGF-I action could influence T3 anabolic effects in osteoblast cell lines.

Normal osteoblasts and MC3T3-E1 osteoblastic cells were used as model systems for our studies because these cells have the capacity to express phenotypic markers of osteoblast function and to synthesize IGF-I under basal and T3-stimulated conditions.^(2,39) We selected [3 H]proline incorporation, OCN production, and ALP activity because these parameters generally are accepted as osteoblast phenotypic markers. We chose to measure 3 H-proline incorporation into total protein and not to distinguish between incorporation into collagenase-digestible protein and noncollagen protein because previous studies have shown that T3 stimulates 3 H-proline incorporation into both collagen and noncollagen protein to a similar extent in bone.⁽⁴⁰⁾

The choice of the cell type to use for a particular parameter was dictated by both cell characteristics and methodological feasibility. Under our experimental conditions, we were able to measure adequately the baseline [3 H]thymidine and 3 H-proline incorporation in both normal mouse osteoblasts and MC3T3-E1 cells and these parameters were responsive to T3 stimulation. Although OCN production could be detected in both cell models after T3 treatment, the response was much greater in normal osteoblasts. OCN levels in untreated cells were within the working range of the RIA in normal osteoblasts only; OCN levels in control of MC3T3-E1 cells were below the limit of detection of the assay. Therefore, the effect of the IGF-I antagonist on T3-stimulated OCN production was studied only in normal osteoblasts. Assessment of the effect of the IGF-I antagonist on ALP activity was limited to MC3T3-E1 cells because no stimulatory effects of T3 on ALP were observed in the normal osteoblasts. In previous studies, the effects of T3 on ALP have been variable, with some studies showing stimulatory effects and others reporting inhibitory or no response. For example, T3 failed to affect ALP activity in a study using UMR-106 cells.⁽⁴¹⁾ In normal rat osteoblastic cells, T3 had dose-dependent biphasic effects on ALP.⁽⁴²⁾

We used three different independent approaches to interfere specifically with the IGF-I actions in bone. Both the neutralizing antibody α IR3 and the IGF-I peptide analogue JB1 act as antagonists to the IGF-I receptor to prevent binding of IGF-I and the AS-ODN prevents the synthesis of IGF-I receptor. Both α IR3 and JB1 were shown to block specifically IGF-I-stimulated [3 H]thymidine incorporation in various cell lines.^(34,35) The inhibitory effects of α IR3 also were seen in our cell models where we observed attenuated [3 H]thymidine incorporation in response to IGF-I stimulation in cells cotreated with the antagonists. The specificity of effects achieved by α IR3 was further confirmed by the inability of a control IgG antibody to attenuate IGF-I stimulation. A third approach was to inhibit IGF-I action by decreasing IGF-I receptor number using AS-ODNs specific for IGF-I receptor mRNA. Experiments using transfection of oligonucleotides were performed only in MC3T3-E1 cells because we were not successful in transfecting normal

osteoblasts. The effectiveness of the AS-ODN to reduce IGF-I receptor protein was demonstrated using Western blotting for the IGF-I receptor. Using multiple approaches to interfere with IGF-I actions made it less likely that the observed responses were results of nonspecific side effects from any one of the antagonists.

Our data demonstrated mitogenic effects of T3. Other studies have shown that T3 has a dose-dependent, biphasic effect on osteoblast growth, with stimulation at low and inhibition at high concentrations.⁽⁴³⁾ T3 elicited significant stimulation of [³H]thymidine incorporation in both normal osteoblasts and MC3T3-E1 cells. IGF-I also stimulated [³H]thymidine incorporation in both models. Consistent with our proposed hypothesis, treatment with neutralizing antibody to IGF-I receptor blocked both IGF-I and T3-stimulated [³H]thymidine incorporation.

Our findings also showed anabolic effects of T3 on the osteoblast systems. T3 treatment caused a significant increase in phenotypic markers of osteoblasts. Normal osteoblasts treated with T3 for 72 h showed significant increases in both ³H-proline incorporation and OCN production. To determine whether endogenously produced IGF-I was necessary for T3-stimulated osteoblastic responses, we tested the effect of a neutralizing anti-IGF-I receptor antibody on T3-induced ³H-proline incorporation and OCN production. In addition, an IGF-I peptide antagonist JB1 was tested for its effect on T3-induced [³H]proline incorporation. Both the antibody and the peptide antagonist had significant attenuating effects on the T3-stimulated responses in normal osteoblasts, indicating a requirement for IGF-I in T3-induced total protein and OCN synthesis. Treatment with α IR3 antibody also attenuated T3-stimulated [³H]proline incorporation in the MC3T3-E1 cells.

Dependence on IGF-I also was observed for T3 effects on ALP activity in MC3T3-E1 cells. Treatment with 10 nM T3 greatly stimulated ALP expression; however, cells transfected with AS-ODN did not show increased ALP activity with the same T3 treatment. It is interesting that even a partial reduction in the expression of IGF-I receptor resulted in a significant attenuation in response. Similar results also were reported in a recent study by Muller et al. using ovarian cancer cells transfected with IGF-I receptor AS-ODN.⁽⁴⁴⁾ They showed that IGF-I receptor protein was reduced by the AS-ODN to 53% of control value and that transfected cells showed decreased proliferation in response to IGF-I stimulation. The control MS-ODN, which we demonstrated by Western immunoblotting to have no effect on IGF-I receptor expression, did not have an attenuating effect on T3-stimulated ALP activity. This result further confirmed that the IGF-I pathway is necessary for T3-stimulated responses.

Given our results showing that agents that interfere with IGF-I actions can block T3-stimulated mitogenic and anabolic responses in osteoblasts, it was expected that treatment with IGF-I alone would increase both mitogenesis and the osteoblast phenotypic markers. Unexpectedly, in both models, treatment with IGF-I alone caused only a stimulation in mitogenic activity and did not produce a stimulation in ³H-proline incorporation, OCN production, or ALP expression; cells that were cotreated with both IGF-I and T3 did not produce responses that were significantly greater than cells treated with T3 alone. These data were unexpected

because previously published studies demonstrated IGF-I to have anabolic effects on bone. IGF-I stimulated both collagen and noncollagen protein production in cultures of fetal rat calvaria and human osteoblasts.^(21,45-47)

A major and the most significant difference between our models and those in which IGF-I alone had effects is that others did not use resin-stripped serum, which we used in all of our anabolic studies. It is possible that other factors could have been present and were removed by the stripping procedure; these factors might complement IGF-I to stimulate anabolic actions in bone. This possibility is supported by the findings of Pirskanen et al., who showed that IGF-I alone stimulated mitogenesis in MG-63 cells but failed to stimulate OCN in the absence of other factors such as calcitriol.⁽⁴⁸⁾ T3 treatment might induce the synthesis of these other factors in its pleiotropic effects on bone.

It may be significant that other studies were performed in different osteoblast systems than those we used. These cell-based differences in response to an IGF stimulus may reflect receptor population and receptor cross-reactivity and may depend on cell type species and osteoblast lineage and maturation.^(49,50) Recent studies have shown differential effects of thyroid hormones on several parameters, including IGF-I, at different skeletal sites.⁽⁵¹⁻⁵³⁾ Malpe et al. further demonstrated differential levels of IGF-I/II and IGF binding proteins (IGFBPs) 3, 4, and 5 at five different skeletal sites.⁽⁵⁴⁾ Because both of the cell models used in our study were calvarial derived, it would be of interest in future studies to determine whether the IGF-I dependence of T3 effects observed in the current study also are found in cells from other skeletal sites.

Interactions with various IGFBPs also can affect the observed responses. Both T3 and IGF-I can increase the production of IGFBPs.⁽²⁴⁾ IGFBPs can modulate IGF actions in bone. Certain IGFBPs inhibit IGF-I actions and others have been shown to have enhancing effects.^(50,55,56) The relative amounts of the IGFBPs produced by T3 and IGF under different experimental conditions and in different models could play a role in the different responses found, because the final osteoblast response depends on presence or absence of endogenous IGFs and other IGFBPs.

In summary, different complementary methods of blocking IGF-I actions all attenuated T3-stimulated responses in osteoblasts. From our findings we conclude that IGF-I is a necessary component for T3-stimulated anabolic osteoblast functions and that the stimulation of IGF-I production by T3 is thus essential for the anabolic effects of the hormone. The observation that IGF-I alone did not elicit the same responses as T3 on ³H-proline incorporation, OCN production, or ALP activity (although it stimulated mitogenesis) suggests that IGF-I is a necessary but not a sufficient factor for T3 responses. Other T3-stimulated local factors that are essential for IGF-I action factors also may contribute to the anabolic effects of T3 on bone.

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Mutual Up-regulation of Thyroid Hormone and Parathyroid

Hormone Receptors in Osteoblastic ROS 17/2.8 Cells

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ABSTRACT

Parathyroid hormone (PTH) and thyroid hormone (T3) stimulate anabolic and catabolic processes in bone predominantly by acting on osteoblasts. Both inadequate and excessive secretion of either hormone can result in clinical bone disorders. In addition, T3 and PTH related peptide (PTHrP) have multiple effects on a wide number of other tissues modulating both cell differentiation and proliferation. To address the question of whether there might be functional mutual regulation of T3 receptors (TR) and PTH/PTHrP receptors (PTHR), we studied their expression and receptor-mediated intracellular effects in osteoblastic ROS 17/2.8 cells. PTHR were up-regulated by T3 pre-treatment (10^{-10} – 10^{-6} M) in ROS 17/2.8 cells in a dose-dependent manner. T3 pre-treatment increased both PTH-induced CREB phosphorylation and PTH-induced intracellular calcium transients, and further decreased PTH-induced down-regulation of alkaline phosphatase activity, suggesting that there are functional consequences of the PTHR up-regulation. Pre-treatment with PTH (10^{-10} – 10^{-6} M) or PTHrP (10^{-9} M) for 3-4 days resulted in a dose-dependent up-regulation of TR in ROS 17/2.8 cells. cAMP analogues or a calcium ionophore were able to mimic the effect of PTH on TR binding, suggesting that either the cAMP-signaling pathway or Ca^{2+} could be involved in PTH-induced up-regulation of the TR. These observations provide a novel example of mutual interactions between nuclear receptors and membrane receptors, and may have significant implications for the regulation of bone remodeling in health and disease.

Introduction

Both thyroid hormone (T3) and parathyroid hormone (PTH) have multiple critical roles in bone. Mice lacking thyroid hormone receptors ($TR\alpha1^{-/-}$, $\beta^{-/-}$) display retarded growth and bone maturation, especially a decrease in longitudinal bone growth (1). In children, hyperthyroidism can cause acceleration of growth and skeletal development (2), whereas hyperthyroidism may accelerate bone loss in adults (3). Conversely, hypothyroidism can be associated with retarded bone maturation and stunted growth. PTH is one of the most important regulators of bone physiology. Hyperparathyroidism can result in bone loss, while low dose and intermittent pulses of PTH stimulate bone formation in animals and humans (4, 5). The fact that both T3 and PTH stimulate both anabolic and catabolic processes in bone raises interesting questions about their possible interactions, and the potential physiologic roles of such coordinate actions. In human subjects the renal response as well as the bone response to administration of PTH is much greater in hyperthyroidism compared to the responses in hypothyroid or euthyroid subjects (6). These clinical observations suggest that PTH and T3 have synergistic actions and result in increased bone resorption. It is noteworthy that more than 20 cases of patients with coexistent hyperthyroidism and primary hyperparathyroidism have been reported (7, 8).

Osteoblasts appear to be the major direct cellular target of PTH and T3 action in bone, since these cells possess both PTH receptors (PTHr) and T3 receptors (TR). $TR\alpha$, $TR\beta$ and PTHr have been characterized in cells of the osteoblast lineage (9, 10).

Other studies documented that T3 stimulates proliferation of rodent and human osteoblastic cells (11, 12), increases alkaline phosphatase activity (13), and increases production of osteocalcin (14), as well as bone collagen and non-collagen proteins (15). PTH increases DNA synthesis and induces the expression of early response genes in osteoblastic ROS 17/2.8 cells (16, 17). In contrast to these stimulatory effects, PTH decreases alkaline phosphatase activity and collagen synthesis (16, 17). These findings suggest that functional interactions between PTH and T3 could exist in osteoblasts.

Many hormones and growth factors likely influence the expression and function of PTH and PTH-related peptide (PTHrP) receptors and certainly of T3 receptors. In the case of the PTHR, glucocorticoids and transforming growth factor- β up-regulate PTHR in ROS cells (18, 19, 20). 1,25-dihydroxyvitamin D, tumor necrosis factor- α and retinoic acid down-regulate PTHR and receptor mRNA expression (21, 22). Insulin and hydrocortisone increase TR α transcripts (23, 24), while interleukin 1 β , interleukin 6 and tumor necrosis factor- α down-regulate TR α and TR β binding capacity in a liver cell line (25).

In addition, both PTH and T3 have been shown to affect the production of a number of growth factors and cytokines in osteoblasts (16, 25, 26). These factors also affect bone remodeling, adding to the potential and complexity of the system. Although it is reasonable to infer that there may be interactions between T3 and PTH in the regulation of these factors, there is only limited information regarding this possibility. The purpose of the current study was to determine whether there are mutual interactions between T3 and PTH at the receptor level in osteoblastic cells, and if so, to examine the mechanisms and consequences of these interactions.

Materials and Methods

Reagents

L-3,5,3'-[¹²⁵I]- triiodothyronine ([¹²⁵I]-T3) were purchased from Dupont, New England Nuclear (Boston, MA), [¹²⁵I]-[Nle⁸,Nle¹⁸,Tyr³⁴] bPTH (1-34) ([¹²⁵I]-PTH) and [α ³²P] deoxy-CTP were from Amersham (Arlington Heights, IL). Bovine PTH (1-34) amide (PTH) was purchased from Bachem Inc (Torrance, CA), Anti-CREB and Anti-Ser¹³³-phosphorylated CREB were from Upstate Biotechnology, Inc (Lake Placid, NY), AMV reverse transcriptase and Taq polymerase were from Promega (Madison, WI). All chemicals were obtained commercially and were of the highest purity available.

Cell culture

ROS 17/2.8 cells were a generous gift from the Endocrine Unit, Massachusetts General Hospital (Boston, MA), initially provided by Dr. Gideon Rodan (Merck Sharp & Dohme Laboratories, West Point, PA). Cells were maintained in Ham's F12 Medium supplemented with 5% fetal bovine serum (FBS) (Gibco Laboratory, Grand Island, NY) with or without resin-stripping in a humidified atmosphere of 95% O₂/ 5% CO₂ at 37° C. Cells were plated at a density of 5 x 10⁴ cells/cm² and experiments were performed with confluent cells in 24-well plates. The medium was changed every other day until the cells reached confluence and daily thereafter.

PTH receptor binding on ROS cell

Confluent ROS cells were maintained in 5% resin-stripped FBS F12 medium. It is necessary to deplete T3 from the supplemented serum for observing the action of T3, since bovine serum contains a significant level of T3 (27). The cells were rinsed initially with buffer containing 50 mM Tris (pH 7.7), 100 mM NaCl, 2 mM CaCl_2 , 2.5 mM KCl, 0.5% heat-inactivated FBS, and 5% heat-inactivated horse serum, and then incubated in this buffer with 2×10^5 cpm/ml of [^{125}I]-PTH, in the presence or absence of unlabeled PTH 10^{-6} M at 16° C for 4h, as previously described (10). For Scatchard analysis, cells were incubated with [^{125}I]-PTH in the presence of increasing concentrations of unlabeled PTH. Incubations were terminated by aspirating the supernatant and washing the cells with ice-cold binding buffer. Cell membrane-bound radioactivity was recovered by lysing the cells with 1 N NaOH, and radioactivity was determined in a gamma counter. Specific binding was determined by subtracting binding in the presence of unlabeled PTH 1 μM from total binding.

Thyroid hormone receptor binding on ROS cells and calvaria

ROS 17/2.8 cells were lysed in 0.5% Triton X 100, 50 mM Tris.HCl, pH 7.5 and nuclear proteins subsequently extracted in 0.4 M KCl, 50 mM Tris.HCl, pH 7.5 (extract buffer). For calvaria experiments, calvaria were dissected and cultured with the treatments. The culture medium was changed daily. After 72 hours, the calvarium was removed and homogenized with a Polytron. The pellet was resuspended and incubated at 4°C for 60 min in extract buffer. It was then centrifuged for 30 min at 13,000 rpm, and the nuclear protein extract collected. Equal amounts of nuclear protein

extracts were incubated with [125 I]-T3 in the presence or absence of unlabeled T3 (4×10^{-6} M) at 4° C overnight, as previously described (28). Free and bound [125 I]-T3 was separated by vacuum filtration through nitrocellulose membranes (Millipore HAWP). Radioactivity on membranes was counted with a gamma counter. Specific binding was determined by subtracting binding in the presence of unlabeled T3 (4×10^{-6} M) from total binding.

Western blot analysis

Cytosolic proteins were extracted in a solution (0.9 M NaCl, 50 mM Na₂HPO₄, 2.5 mM EDTA, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 50 mM HEPES-Tris, pH 7.4) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of cellular protein extracts were resolved by 10% SDS-PAGE and transferred onto nitrocellulose filters. In each experiment, duplicate membranes were prepared. The membranes were incubated with 3% nonfat milk in PBS for 1.5 h and then incubated overnight at 4° C with rabbit polyclonal antibodies against either total CREB or Ser¹³³-phosphorylated CREB. Immunoreactive proteins were detected using an anti-rabbit horseradish peroxidase-conjugated antibody and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL) as previously described (29). Antibody-antigen complexes were detected with Kodak X-Omat film and quantified with a densitometer.

RNA isolation, RT-PCR analysis

Cells were extracted in 4 M guanidine isothiocyanate . Total RNA was separated

by ultracentrifugation. The pellet was solubilized in 0.1% SDS, precipitated with ethanol, and reconstituted in DEPC-H₂O, characterized by agarose gel electrophoresis, and quantitated by spectrophotometer at A₂₆₀. The RT-PCR assay methodology has been previously described in detail (30). In brief, one µg of RNA product was reverse transcribed using random hexamer priming. The cDNAs were then amplified by PCR in the presence of [α^{32} P]deoxy-CTP using primers for the PTH receptor (5'GTTGC-GCGTGCAGTGCAGCCGCCTAAAGTA3' and 5'GTGGATGCAGATGACGTCA-TGACTAAAGAG3') as well as cyclophilin as an internal control. PCR reactions were separated on acrylamide gels and quantitated by a BAS 1000 phosphoImager (Fuji photo Film Co, LTD, Japan). The results are expressed as a ratio of Integrated Optical Density of PTHR to cyclophilin.

Assay for alkaline phosphatase activity and protein concentration

For measurement of intracellular alkaline phosphatase activity, cells were first washed with PBS and then ruptured by repeated freeze-thaw cycles and sonication. Alkaline phosphatase activity was measured by the p-nitrophenyl phosphate method (31). Protein concentration was measured by the BioRad protein assay (BioRad Laboratories, Inc, Hercules, CA)

Intracellular calcium measurements

Cells were harvested, washed and suspended in a loading buffer consisting of 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, 1 mM CaCl₂, and 1% bovine serum albumin, pH 7.4. Cells were incubated with 2 µM fluo-3 AM

(Molecular Probe, Eugene, Oregon), by gentle shaking for 30 min in loading buffer at room temperature. The cells were centrifuged, washed and resuspended in loading buffer at a concentration of approximately 10^6 cells/ml. A Perkin-Elmer LS-5 luminescence spectrophotometer (Foster City, CA) was used for fluorometric determinations (excitation/emission: 505/530 nm). After a 1-min base-line recording, test solutions were added and the maximal fluorescence was measured. The intracellular calcium concentration in nM quantities were calculated by the following formula:

$[Ca^{2+}]_i = K_d \times (F - F_{min}) / (F_{max} - F)$, where K_d is the dissociation constant for fluo-3 (400 nM), F_{max} is the maximal fluorescence measured by addition of digitonin (40 μ M), F_{min} is the fluorescence of cell suspension without fluo-3 loading, and F is the measured fluorescence of sample in the cuvette.

Statistical analysis, binding parameters

Data are means \pm SEM. Statistical significance was determined by ANOVA and post-test. Differences were considered significant at $P \leq 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***). Parameters of binding were determined from the Scatchard plots using the Prism Graph Pad software (San Diego, CA).

Results

Effect of T3 on [¹²⁵I]-PTH binding

Pre-treatment of ROS 17/2.8 cells with T3 (10^{-10} – 10^{-6} M) in F12 medium supplemented with 5% resin-stripped serum for 3 days resulted in a biphasic dose-dependent increase in [¹²⁵I]-PTH(1-34) binding compared with binding in the vehicle-treated control cells (Fig. 1A). Maximal specific binding (144% of control) was seen with 10^{-7} M T3. The time point for measurement was selected from a time-course experiment (Fig. 1B), which demonstrated that the effect on binding started from day 1 and reached a plateau (134% of control) after three days of incubation with T3 (10^{-8} M).

Scatchard analysis showed that T3 increased [¹²⁵I]-PTH binding sites by approximately 38%, from 42,963 binding sites/cell (control) to 59,441 binding sites/cell (T3-treated). The binding affinity was changed minimally (Fig. 1C). There was no significant difference in cell numbers after pre-treatment of ROS 17/2.8 cells with T3 (10^{-10} – 10^{-6} M) for 3 days (Fig. 1D). Therefore, the enhanced binding was due principally, if not totally, to an increase in available PTH receptors, rather than to altered receptor-binding affinity or cell proliferation.

Effect of T3 on PTH receptor mRNA expression

RNA was extracted from control ROS 17/2.8 cells and cells treated with T3 (10^{-7} M) for 3 days, respectively. RT-PCR analysis revealed that the level of the PTH receptor transcripts was increased 44% by T3 as compared with the control (Fig. 2).

Effect of T3 on PTH receptor function

PTH induces intracellular calcium transients in osteoblastic cells (32). The increase reaches a peak within 15 sec. Pre-treatment of ROS 17/2.8 cells with T3 (10^{-8} M) for 3 days potentiated the calcium signal elicited by PTH (4×10^{-9} M) (Fig. 3A). The difference in the $[Ca^{2+}]_i$ response was 34 nM (48 nM in T3-treated; 14 nM in control). The increase in PTH-induced intracellular calcium signals is consistent with the up-regulation of the PTH receptor by T3.

PTH induces phosphorylation of CREB through cAMP-dependent protein kinase A (33). Pre-treatment of ROS 17/2.8 cells with T3 (10^{-8} M) for 3 days, followed by a 10 min pulse of PTH (10^{-8} M) potentiated PTH-induced CREB phosphorylation, increasing by 10 fold compared to controls that were not treated with T3 (Fig. 3B). It is likely that the enhanced CREB phosphorylation is a consequence of the T3 stimulated up-regulation of the PTHR.

T3 increases alkaline phosphatase activity and PTH down regulates alkaline phosphatase activity in ROS 17/2.8 cells (13,17). After pre-treatment of ROS 17/2.8 cells with T3 (10^{-8} M) or/and PTH (10^{-8} M) for 3 days, the alkaline phosphatase activity of PTH-treated cells decreased ($34.9 \pm 0.8\%$ of control). Co-treatment with PTH and T3 resulted in a further decrease to $15.5 \pm 3.6\%$ compared to the control cells (Fig. 3C).

Effect of PTH/PTHrP on $[^{125}I]$ -T3 binding

Pre-treatment of ROS 17/2.8 cells with PTH (10^{-10} – 10^{-6} M) for 4 days resulted in a dose-dependent increase in $[^{125}I]$ -T3 binding compared with the controls (Fig. 4A).

Maximal specific binding in cells treated with PTH (10^{-6} M) was 314% of that seen in controls. The time course of the effect of PTH (10^{-8} M) on [125 I]-T3 binding showed a maximal effect (245%) on day 3 (Fig. 4B). Scatchard analysis of the binding data indicated that the apparent binding affinity for T3 was similar in PTH-treated and control cells. Calculation of the number of binding sites per cell revealed that there was approximately 48% increase in the number of T3 binding sites in PTH-treated cells compared to the control cells (7,845 binding sites/cell in the controls; 11,629 binding sites/cell after PTH treatment) (Fig. 4C). Pre-treatment of ROS 17/2.8 cells with PTHrP (10^{-9} M) for 4 days also increased [125 I]-T3 binding to 155% compared to the controls (Fig. 4B). Since the PTH receptor studied could be activated by both ligands (PTH and PTHrP), it is likely that the relevant receptor is the PTH/PTHrP receptor (35).

Effect of PTH/PTHrP on [125 I]-T3 binding in calvaria

PTH treatment not only increased [125 I]-T3 binding in the osteoblastic cell line, but also in mouse calvaria (Fig. 5). This is logical in that the osteoblast is probably the major cell type in the calvaria. For these studies, mouse calvaria were pre-treated with PTH (10^{-8} – 10^{-6} M) or PTHrP (10^{-8} M) for 3 days. [125 I]-T3 binding was increased to 353% and 174% respectively compared to the controls.

cAMP analogues or calcium ionophore can mimic PTH effects on TR binding

Several signal transduction pathways are activated by ligand binding to the PTH/PTHrP receptors. The predominant responses in these cells are the activation of

adenylate cyclase and increased intracellular calcium. As shown in Fig. 6A, both forskolin, a potent activator of adenylate cyclase, and 8-Br-cAMP, a cAMP analogue, were able to increase [125 I]-T3 binding in ROS 17/2.8 cells. Also (Fig. 6B) ionomycin, a calcium ionophore, increased [125 I]-T3 binding in ROS 17/2.8 cells, whereas phorbol-12,13-dibutyrate (10^{-7} M), a protein kinase C stimulator, had no effect. In other studies concentrations of 10^{-9} - 10^{-6} M phorbol-12,13-dibutyrate were also ineffective (data not shown). This suggests that either the cAMP pathway or Ca^{2+} signaling may be involved in the PTH induced up-regulation of TRs.

Discussion

The intricate balance of concomitant bone resorption and formation is critical for the maintenance of bone metabolism and structure. Both processes are dependent on the action of osteoblasts and regulated by signals from systemic hormones and locally-generated growth factors and cytokines. Among them, PTH and T3 play a particularly important role. The endocrine system offers a number of examples of mutual regulation of peptide and steroid hormone receptors. For example, thyroid hormone treatment has been shown to increase mRNA for cardiac membrane β -adrenergic receptors, thereby potentially augmenting the cardiac responses to catecholamines (34). Glucocorticoids up-regulate, and 1,25-dihydroxyvitamin D down-regulates PTH receptors in osteoblastic cells (19, 21). Because of the influence of both the T3 and PTH/PTHrP receptor pathway on bone biology, we addressed the question of whether these hormone systems modulate each other in bone cells. This study provides evidence

for a positive regulation of the PTH/PTHrP membrane receptors by T3, and of T3 nuclear receptors by PTH. This illustrates that these hormones can act indeed in a synergistic fashion.

Our results indicate that PTHR_s were up regulated by T3 pre-treatment (10^{-10} – 10^{-6} M) in osteoblastic ROS 17/2.8 cells (Fig 1A, 2). The minimal effective dose of T3 was 10^{-10} M. Since 99.5% of T3 is normally bound to serum protein, after equilibrium the free T3 concentration in resin-stripped serum medium would be less than 10^{-11} M, which is in the human physiological range. Moreover, T3 pre-treatment increased both PTH-induced intracellular calcium transients and PTH-induced CREB phosphorylation (Fig 3A, 3B), possibly through up-regulation of PTHR by T3 treatment. In ROS 17/2.8 cells T3 enhances and PTH decreases alkaline phosphatase activity, and co-treatment of PTH/T3 results in augmented decrease of enzyme activity (Fig 3C). This suggested that the effect of PTH on alkaline phosphatase activity is predominantly receptor mediated, and that T3-induced up-regulation of PTHR_s results in potentiation of the suppressive effect of PTH on alkaline phosphatase. Since T3 can stimulate cell proliferation in certain circumstances, the number of cell membrane receptors could be increased by this mechanism. Although [3 H]-thymidine incorporation was slightly increased in T3-treated cells (data not shown), there were no significant changes in cell number when cells were cultured in medium containing resin-stripped serum (Fig 1D). Therefore, the enhanced [125 I]-PTH binding was due principally, if not totally, to an increase in available PTH receptors, rather than to altered receptor-binding affinity or cell proliferation.

T3 receptors were up-regulated in ROS 17/2.8 cells after pre-treatment with PTH

(10^{-10} – 10^{-6} M) for 3-4 days in a dose-dependent manner (Fig 4). This up-regulation of TR binding was also seen after PTHrP (10^{-9} M) treatment. This suggests that the effects are mediated through a PTH/PTHrP receptor, i.e. a type 1 receptor. As of now, three subtypes of PTHRs have been characterized; type 1 is activated by both PTH and PTHrP; type 2 is activated by PTH only; and type 3 is activated by PTHrP only (35).

Pre-treatment with either PTH or PTHrP likewise increases [125 I]- T3 specific binding in mouse calvaria (Fig 5) and osteoblastic UMR106 cells (data not shown). This demonstrates that PTH/T3 interactions were not restricted to ROS 17/2.8 cells, but also occur in other cells of the osteoblast lineage. Both cAMP analogues and the calcium ionophore, ionomycin, were able to mimic PTH effect on TR binding (Fig 6 A,B), suggesting that either the cAMP-signaling pathway or increases in intracellular calcium could mediate PTH-induced up-regulation of TR.

Scatchard analysis showed that T3(10^{-8} M) and PTH(10^{-8} M) increased the binding sites for the other hormone by approximately 38% and 48% per cell, respectively. This increase could be at the level of transcriptional regulation of the receptors. Regulation of PTH/PTHrP and TR receptor binding and mRNA expression by other hormones have been reported. PTH/PTHrP receptor availability was up-regulated (87%) by hydrocortisone (2×10^{-7} M), and simultaneously the level of receptor transcripts were also increased in ROS 17/2.8 cells (18,19). In the case of the TR, insulin up-regulated nuclear thyroid hormone binding (60%), and increased TR α mRNA expression in bovine aortic endothelial cells(23).

The mechanism of T3/PTH interactions is still unclear. The TR α 1 promoter contains three putative CRE and one putative TPA site(36), and TR β 1 promoter contains one

putative TRE (37). All of these elements could be potentially regulated by PTH through phosphorylation of CREB, TR, AP1 or other TRAPs (Thyroid Receptor Auxiliary Protein) and coregulators including calmodulin. TR not only can bind to TRE but also can interact with Jun and Fos (38) as well as with RXR, which are potential targets for TR activation of PTHR expression, since the PTHR promoter contains putative AP-1 and RXR β sites(39).

In conclusion, the present experiments demonstrate that PTH treatment increases the number of thyroid hormone receptors in osteoblastic ROS 17/2.8 cells in a time- and dose-dependent manner. Conversely, T3 treatment increases the number of PTH receptors and receptor transcripts in ROS 17/2.8 cells as well. This is a novel example of mutual regulation between nuclear receptors and membrane receptors, an observation that has significant implications for the regulation of bone remodeling. Further characterization of the involved TR isoforms, the signaling pathways, and the regulation of the promoter regions of both the PTH/PTHrP receptors and TRs may shed further light on these interactions.

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FIGURE LEGENDS

Fig. 1. Effect of T3 on [125 I]-PTH specific binding in ROS 17/2.8 cells. Confluent cells were incubated in F12 medium supplemented with resin-stripped FBS for two days, then treated with different doses of T3 or vehicle (C) for three days respectively. Media were renewed daily. PTH receptor binding was then assayed as described in Materials and Methods. Statistical significance was determined by ANOVA and post test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A. Dose response curve of effect of T3 (10^{-10} - 10^{-6} M) and vehicle (C) on [125 I]-PTH specific binding. Data are means and SEM of $n = 3$ and are representative of two experiments, both of which gave similar results. **B.** Time course of effect of T3 (10^{-8} M) and vehicle (C) on [125 I]-PTH specific binding. Data are means and SEM of $n = 4$. **C.** Scatchard plot of effect of T3 (10^{-8} M) and vehicle(C) on [125 I]-PTH specific binding. Cells were incubated with [125 I]-PTH in the presence of increasing concentrations of unlabeled PTH for 16 h at 4°C . PTH receptor binding was then determined. B/F: Bound to Free ratio. Data are from two experiments. **D.** Dose response curve of effect of T3 (10^{-10} - 10^{-6} M) and vehicle (C) on cell growth. Cells were trypsinized and counted at the end of a 3-day treatment. Data are means and SEM of $n = 4$.

Fig. 2. RT-PCR of PTH receptor mRNA expression in ROS 17/2.8 cells.

Confluent cells were treated with T3 (10^{-7} M) or vehicle (C) in F12 medium supplemented with resin-stripped FBS for three days. Cell lysate RNA was extracted.

RT-PCR was performed as described in Materials and Methods. PTHR primers were used to amplify the PTH receptor cDNA. Primers for cyclophilin were used as internal standards. (A) Representative autoradiograph (The lanes labelled 3 and 7 denote the cDNA sample volume (μ l) used for PCR); (B) Quantitative results expressed as arbitrary units of Integrated Optical Density (IOD) calculated as a ratio of the expression of PTH receptor (PTHR) to cyclophilin.

Fig. 3. T3 pre-treatment enhances PTH-induced functional response in ROS 17/2.8 cells. Confluent cells were incubated with F12 medium supplemented with resin-stripped FBS for two days, then treated with T3 (10^{-8} M), PTH (10^{-8} M) or vehicle (C) for three days respectively. Media were renewed daily. **A.** Effect of T3 on PTH induced intracellular calcium transients. Cells were trypsinized, centrifuged and resuspended in loading buffer with 2 μ M fluo-3 AM. Calcium transients were elicited by PTH (4×10^{-9} M) as described in Materials and Methods. **B.** Effect of T3 on PTH-induced CREB phosphorylation. Cytosolic proteins were extracted and Western blot analysis was performed with antibodies against pCREB or total CREB respectively as described in Materials and Methods. The ratio of pCREB to total CREB was determined using densitometry and expressed as arbitrary units of Integrated Optical Density (IOD). The data are the representative of two experiments. **C.** Effect of PTH and/or T3 on alkaline phosphatase activity. Confluent cells were either treated with PTH (10^{-8} M), T3 (10^{-8} M), PTH (10^{-8} M) plus T3 (10^{-8} M) or vehicle for three days respectively. Alkaline phosphatase activity was then assayed as described in Materials

and Methods. Data are means and SEM of $n = 4$ and are representative of two experiments.

Figure 4. Effect of PTH on [125 I]-T3 specific binding in ROS 17/2.8 cells.

Confluent cells were incubated in F12 medium and treated with different dose of PTH or vehicle (C) for four days respectively. Media were renewed daily. T3 receptor binding was then assayed as described in Materials and Methods. Statistical significance was determined by ANOVA and post test. * $P < 0.05$; ** $P < 0.01$;

*** $P < 0.001$.

A. Dose response curve of effect of PTH (10^{-10} - 10^{-6} M) and vehicle (C) on [125 I]-T3 specific binding. Data are means and SEM of $n = 3$. B. Time course of effect of PTH (10^{-8} M), PTHrP (10^{-8} M), and vehicle (C) on [125 I]-T3 specific binding. Data are means and SEM of $n = 2-4$ determinations. C. Scatchard plot of effect of PTH (10^{-8} M) on [125 I]-T3 specific binding. Cells were incubated with [125 I]-T3 in the presence of increasing concentrations of unlabeled T3 overnight at 4°C . T3 receptor binding was then assayed as described in Materials and Methods. B/F: Bound to Free ratio.

Figure 5. Effect of PTH/PTHrP on [125 I]-T3 specific binding in mouse calvaria.

Eight days old mice calvaria were incubated in DMEM containing 10% heat-inactivated horse serum and treated with different doses of PTH (10^{-8} - 10^{-6} M), PTHrP (10^{-8} M) or vehicle (C) for three days respectively. Media were renewed daily. Bone was homogenized and cytosolic proteins were extracted. T3 receptor binding was then

assayed as described in Materials and Methods. Data are means and SEM of $n = 3$ and are representative of two experiments. Statistical significance was determined by ANOVA and post test. ** $P < 0.01$; *** $P < 0.001$.

Figure 6. Factors effect on [125 I]-T3 specific binding in ROS 17/2.8 cells.

A. Effect of forskolin or Br-cAMP on [125 I]-T3 specific binding in ROS 17/2.8 cells.

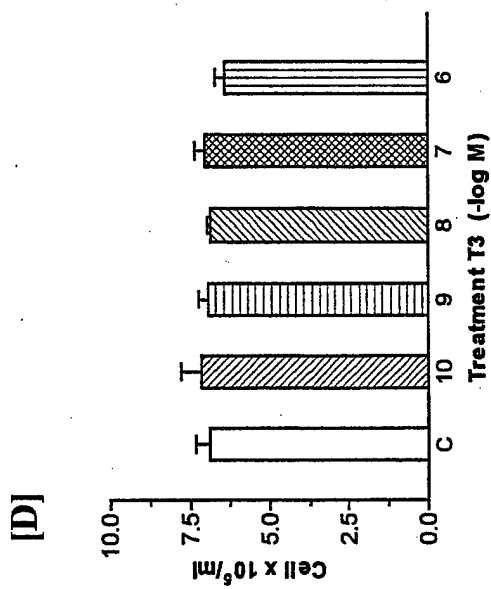
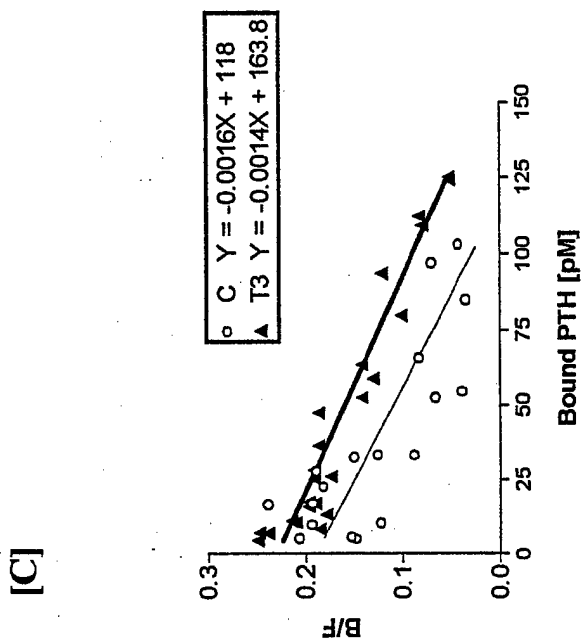
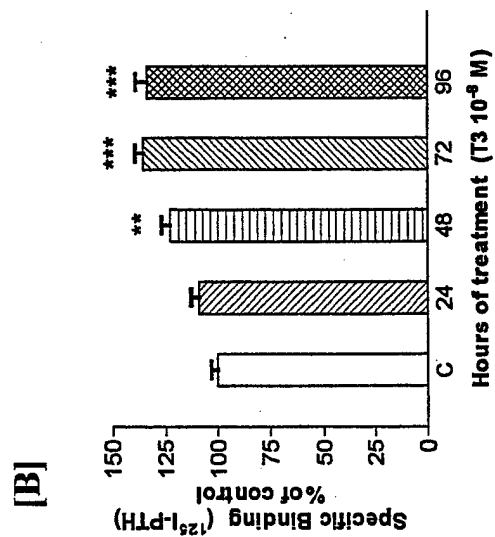
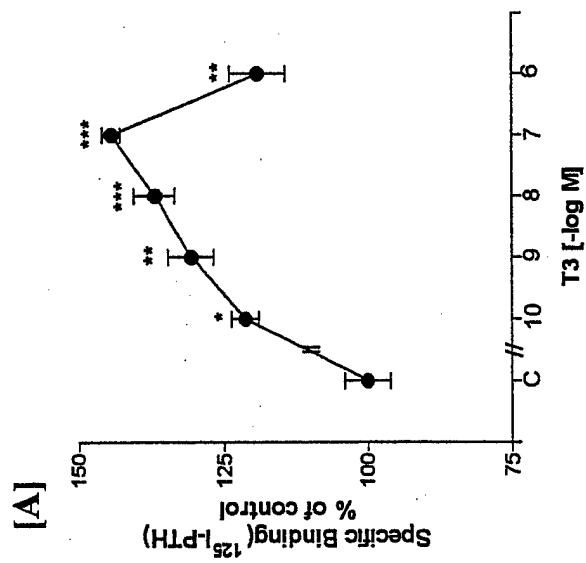
Confluent cells were incubated in F12 medium containing either 10 μ M forskolin, 100 μ M Br-cAMP, or 10^{-8} M PTH for three days. Media were renewed daily. T3 receptor binding was then assayed as described in Materials and Methods. Data are means and SEM of $n = 3$ and are representative of two experiments. Statistical significance was determined by ANOVA and post test. ** $P < 0.01$; *** $P < 0.001$.

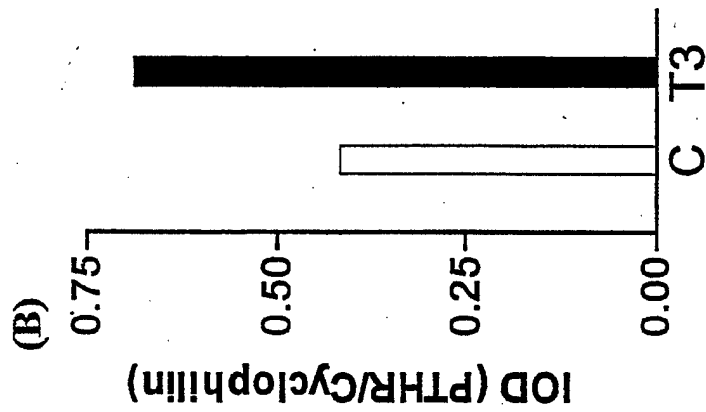
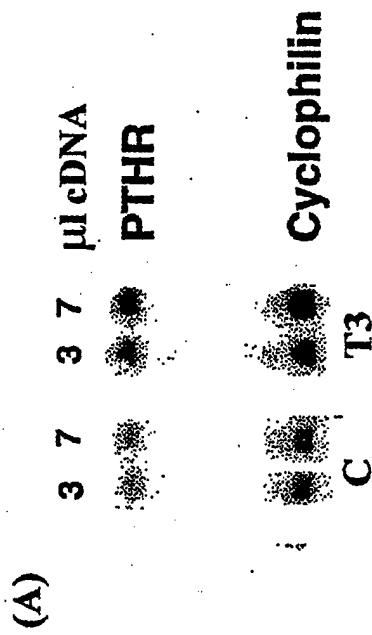
B. Effect of phorbol-12,13-dibutyrate and ionomycin on [125 I]-T3 specific binding in ROS 17/2.8 cells. Confluent cells were incubated in F12 medium containing either 10^{-7} M phorbol -12,13-dibutyrate (PDBu) or 10^{-6} M ionomycin (Iono) for four days. Media were renewed daily. T3 receptor binding was then assayed as described in Materials and Methods. Data are means and SEM of $n = 3$ and are representative of Two experiments. Statistical significance was determined by ANOVA and post test.

* $P < 0.05$; ** $P < 0.01$

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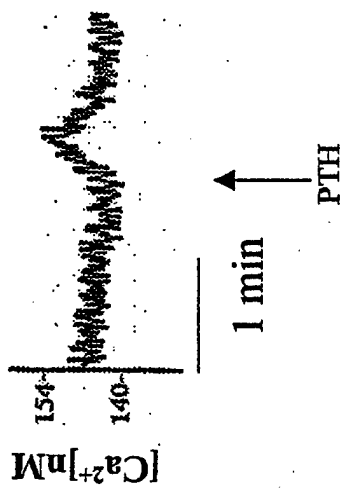
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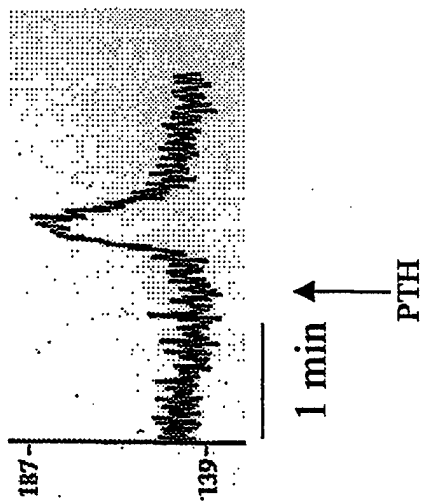


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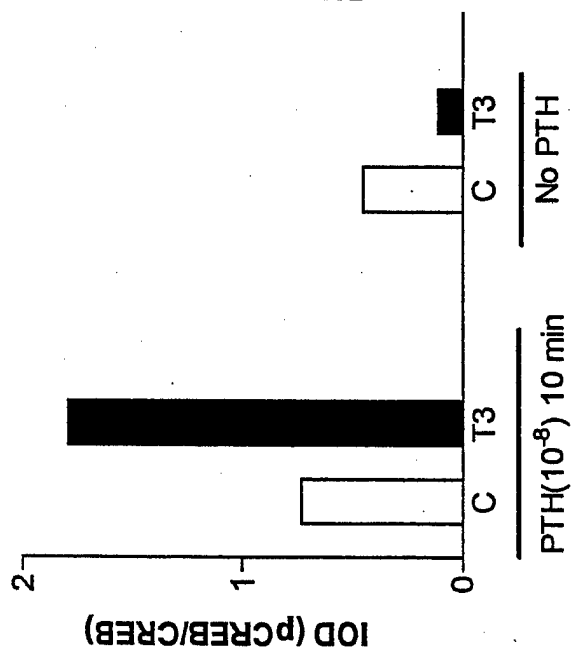
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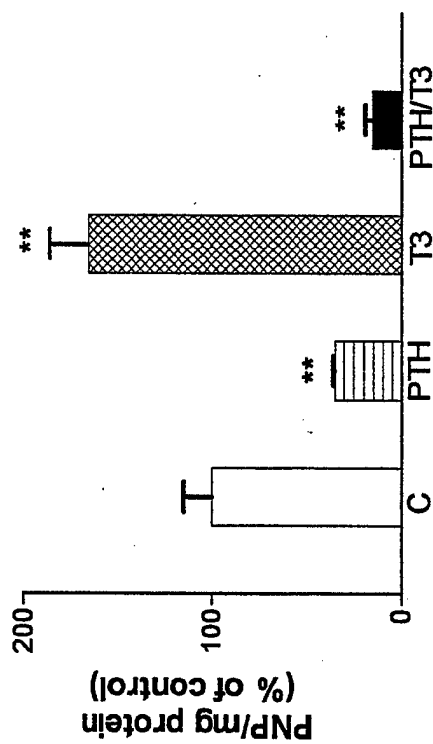
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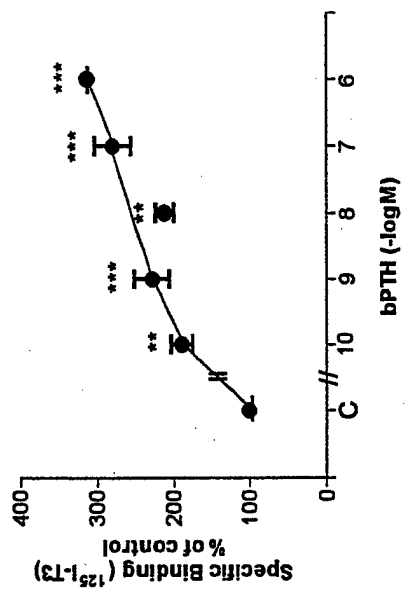
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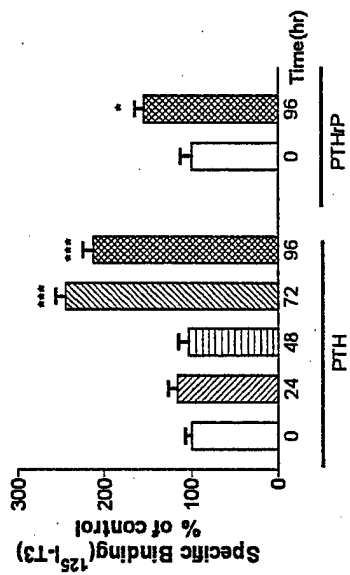
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